(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)
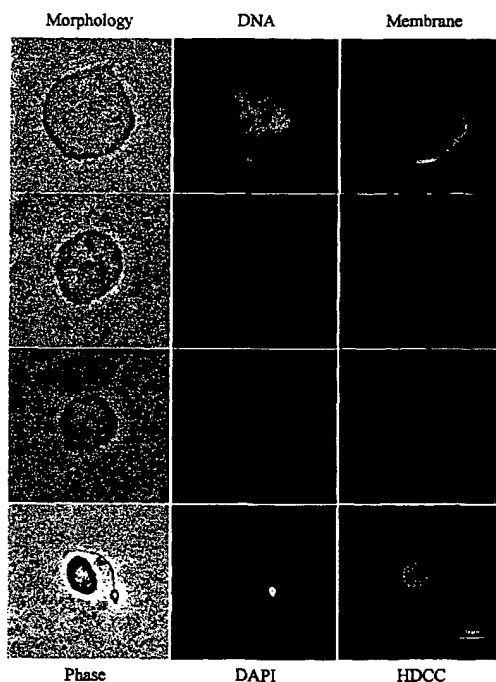
(72) Inventors; and
(75) Inventors/Applicants (for US only): BANERJEE, Subhasis [GB/GB]; St. George's Hospital Medical School, Cranmer Terrace, London SW17 0RE (GB). NARGAND, Geeta [GB/GB]; St. George's Hospital Medical School, Cranmer Terrace, London SW17 0RE (GB). CAMPBELL, Stuart [GB/GB]; St. George's Hospital Medical School, Cranmer Terrace, London SW17 0RE (GB).

(74) Agents: GARDNER, Rebecca et al.; Frank B. Dehn & Co., 179 Queen Victoria Street, London EC4V 4EL (GB).

[Continued on next page]

(54) Title: MANIPULATION OF SPERMATOZOA

Morphology | DNA | Membrane

Phase | DAPI | HDCC

Human sperm dispersion, genomic reprogramming and reassambly of nuclear envelope.

(57) Abstract: The present invention relates to a method of sperm permeabilisation comprising: (a) washing a sample containing sperm in a sperm nuclear extraction buffer (SNEB) which includes one or more metal chelating agents and which is substantially free of non-ionic detergents and divalent cations; (b) contacting the sperm with one or more permeabilising agents; and (c) optionally separating the sperm tail from the sperm head; and to a method of sperm transformation which method comprises incubating a sample of sperm or sperm nuclei in one or more cell-free extracts. Also described are extracts and buffers for use in these methods, methods of preparing the extracts and uses of the transformed sperm nuclei.

WO 02/24872 A2

MW, MX, MZ, NO, NZ, PH, PL, PT, RO, RU, SD, SE, SG, SI, SK, SK (utility model), SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

(84) **Designated States** *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

- 1 -

Manipulation of spermatozoa

5   The present invention relates to the preparation of
       sperm nuclei for analysis or interaction with other
       cells or media. More particularly, the invention
       relates to a method of sperm nuclei permeabilisation, to
       a method of transforming sperm nuclei to a state capable
10   of fusing with the nuclei of other cells and to those
       fusion methods. The invention also relates to media for
       use in said method of transformation and methods for the
       preparation of such media.

15   A few decades ago, while the global human population was
       increasing at an alarming rate, family planning and
       birth control measures were encouraged throughout the
       world, especially in the underdeveloped nations. These
       measures were effectively implemented with the help of
20   hormone-based female contraceptives and occasionally by
       male vasectomy. While public awareness of
       overpopulation, reproductive health and birth control
       measures has increased considerably in recent years,
       several unforeseen and yet serious problems have
25   emerged. Currently, the ability of human couples to
       reproduce is on the decline. These trends appear to
       coincide with a sharp drop in the mean sperm count of
       fertile men from 113 million per ml in 1940 to 66
       million per ml in 1990. About 25% of the failed
30   fertilisation or conception could be accounted for by
       the variety of defects in human spermatozoa. The
       overall increase in infertility or subfertility in
       humans could be related to demographic changes including
       prolonged exposure to industrial pollution, lifestyle
35   factors and an increase in the age of couples willing to
       reproduce.

- 2 -

Not too long ago, the social burdens of infertility, especially in the underdeveloped nations, were invariably carried by women. However, a few important breakthroughs in the last 25 years have led to the

5   conclusion that male and female infertility occur in almost equal proportions in human populations. The causes of infertility are diverse, some are well established, some are controversial. Despite extensive diagnostic testing for azoospermia, for obstructive or

10   unobstructive-oligospermia, ovulatory dysfunction, inflammatory tubal disease, endometriosis and varicocele, the etiology of at least 10% of the human infertility is unknown. Conventional physical parameters such as the measurements of volume of the semen, sperm

15   concentration, morphology and motility used for evaluating the semen quality appears to be insufficient for the diagnosis of male infertility.

Much of male infertility could be accounted for by

20   functionally defective spermatozoa (cell biological) and aberrant spermatogenesis (genetic). About 25-40% of infertile men have high levels of reactive oxygen species (ROS) in the semen leukocytes and in the spermatozoa. These superoxides are generated by

25   hyperactivation of oxidative metabolic pathways in response to the increased cellular levels of reduced cofactor NADPH and H_2O_2. In addition to peroxidative damage of the plasma membrane which affects motility, capacitation and fertility of the sperm cells, the ROS

30   also induces extensive damage and cross-linking of the sperm chromosomal DNA (Twigg, J.P. et al., Hum. Reprod. 13: 429-436, 1998). Such oxidative DNA damage of the spermatozoa has been strongly correlated with childhood cancers.

35

It should be noted that herein the terms sperm and spermatozoa are used inter-changeably.

- 3 -

Currently, the extent of sperm DNA damage is measured by
autoradiography of radioactively labelled nicked DNA, by
alkaline gel electrophoresis or by a single-cell 'comet
assay' where the alkali-denatured damaged DNA migrates
5   away from the sperm head in the gel generating a smear
which appears like a comet. In addition to its semi-
quantitative nature, the comet assay provides no
information on whether genes vital for fertilisation and
embryogenesis are disrupted by ROS in infertile
10   patients. Given the unique organisation of the genome
in human spermatozoa, one can not assume that even 50-
70% of the DNA damage would affect all the genes (<5-10%
of the total genome) needed for fertilisation and
development. Moreover, much of the damage could be
15   repaired by highly efficient DNA-repair mechanisms
operating in the egg (Twigg, J.P. et al., Hum. Reprod.
13: 1864-1871, 1998).

The chromosomal abnormalities in male infertility
20   include aneuploid (47,XYY and 47, XXY, Klinefelter's
syndrome), X- or Y-autosome translocations and deletion
of the Y chromosome. A detailed cytogenetic survey of
azoospermic and oligospermic men revealed a strong
correlation between infertility and abnormalities of the
25   sex chromosome Y. Such abnormalities are represented by
extensive deletions in the long arm of the chromosome Y
(Yq11). Since chromosome Y is the smallest (2% of the
haploid genome) of the 24 human chromosomes, a
comprehensive physical map of the chromosome and >100
30   STSs became readily available. This information
facilitated further analysis of microdeletions in
azoospermia/oligospermia and resulted in the
identification of three loci AZFa, AZFb and AZFc
containing a number of genes which are specifically
35   expressed in the testis for normal spermatogenesis and
are called 'male factors' (Vogt, P.H., Mol. Hum. Reprod.
4: 739-744, 1998). Some candidate genes at Yq11

- 4 -

definitively shown to regulate male fertility have been
identified. Most thoroughly studied candidate genes for
male infertility are DFFRY(Y chromosome RNA recognition
motif) at AZFb locus and DAZ (deleted in azoospermia)
5   located at AZFc. Although DAZ and DFFRY belong to
different gene families, they have remarkable functional
similarities.

An *in vitro* system for analysis of human sperm
10   chromosomes is urgently needed for understanding the
effect of DNA damage and sperm chromosomal abnormalities
on male infertility. Currently, this is accomplished by
microinjection of human sperm into hamster eggs; only a
few laboratories in the world perform this test. Unlike
15   hamster eggs microinjected with sperm, the methods
described herein are inexpensive, very rapid and do not
require animals. Therefore, a large number of patients
can be assessed and counselled within a short period
prior to ICSI or other fertility treatments.
20

In addition to the investigation on male infertility,
the methods can be widely applied in "Genetic risk
assessment" and 'Occupational monitoring'. A
significant number of people (1 in 200) in the human
25   population are carriers of genetic defects which are
manifested at the chromosomal level. These genetic
lesions in the germ-lines arise more frequently among
industrial workers exposed to ionizing radiation or
radiomimetic agents and genotoxic environmental
30   chemicals including >30% licensed pesticides. It is
envisaged that many government organisations and
companies would be keen to use a system to monitor the
chromosomes of workers exposed to potentially hazardous
agents.
35

As well as diagnostic applications, the technology of
the present invention provides a new method of *in vitro*

- 5 -

fertilisation (IVF). Currently, one in five couples
have difficulties with reproduction or are unable to
conceive without medical help. Intracytoplasmic sperm
injection (ICSI) is the most modern, effective treatment
of infertility and has revolutionized the management of
infertility cases in IVF clinics. In the ICSI
techniques, a sperm is injected into the cytoplasm of an
oocyte (for a review, see Tesarik and Mendoza, BioEssays
[1999], 21, 791-801). Before the advent of ICSI,
couples declared infertile because of a male factor were
probably unlikely to have children. While ICSI
continues to be a key therapeutic measure, the possible
genetic risks of passing on damaged genes and defective
chromosomes of the father to the embryo, resulting in
first trimester pregnancy losses, congenital
abnormalities, premature child birth and early childhood
cancers are of serious concern. Unfortunately, the
negative impact of ICSI on the genetic constitution of a
population has never been evaluated by animal
experimentation. Such studies in humans are
impractical, if not impossible, primarily due to ethical
considerations.

The sperm-egg fusion experiments proposed here could be
optimised to establish the genetic risks of infertile
males prior to fertilisation by sperm-somatic cell
fusion and subsequent cytogenetic analysis of metaphase
chromosomes.

The karyotyping of a significant number of sperm
chromosomes by microinjection of zona-free hamster eggs
with human spermatozoa and subsequent cytogenetic
analysis is restricted to a few laboratories in the
world. Pre-implantation diagnosis is restricted to a
very limited number of diagnostic laboratories. The
present invention also offers an alternative to ICSI
which significantly reduces the possibility of passing

- 6 -

on damaged genes and defective chromosomes to the eggs.
This is because the egg ooplasm from high age-group
women exhibits reduced competence in the process of DNA
repair during fertilisation and blastocyst development.
5   The *in vitro* repair of damaged genes of the infertile
sperm in cell-free extracts and subsequent micro-
injection of such sperm nuclei to the oocyte could
increase the efficiency of implantation and thereby
might increase the chances of development of the embryo
10   to term.

The technologies for fusion of two somatic cells by
viral glycoproteins or chemicals (polyethylene glycol)
or by transient electrical pulses have been developed
15   over the last 20-30 years and are routinely used for
producing somatic hybridomas or in a variety of cell
biological and molecular research. However, existing
technologies are insufficient to carry out this kind of
fusion of sperm with somatic cells or with oocytes.
20   This is because the fundamental necessities for *in vitro*
cell fusion are that participating cells have similar
morphology and membrane bilayer structures. Unlike
somatic cells or oocytes, the mammalian spermatozoa are
unique germ cells. For example, a) the human sperm is
25   about one sixth the size of normal cells (for instance
hepatocytes) and about one-fortieth the size of a mature
human oocyte; b) unlike any other cells, the chromosomes
in the sperm are super-condensed (almost crystallized)
by special proteins only produced by males in the
30   testis; and c) spermatozoa can neither divide nor
transcribe their genome (no visible chromosomes by
staining). These characteristics, despite rapid
advancement of biotechnology in the past decade, have
prevented cell fusion as well as hindering genetic or
35   molecular research involving mammalian spermatozoa.

An especially challenging aspect of sperm- somatic cell

- 7 -

or oocyte fusion research is therefore transformation of
the spermatozoa to cells which are morphologically and
structurally similar to those of somatic cells or
oocytes or at least similar enough to fuse with these
5   cell types in vitro.

There are three kinds of system for sperm dispersion and
nuclear assembly reported in the literature, relating to
Xenopus (frog)oocyte (Lohka, M.J and Masui, Y. J. Cell
10   Biol. 98: 1222-1230, 1984), sea urchin egg (Collas,P and
Poccia, D. Therogenology, 49: 67-81, 1998) and fruit fly
(Drosophila) embryo (Berrios, M and Avillion, A.A. Exp.
Cell Res. 191: 64-70, 1990) extracts. These extracts
prepared by releasing materials from mature eggs or
15   fruit fly embryos by high speed ultra centrifugation,
are capable of dispersing corresponding sperm nuclei
(frog, sea urchin or fruit fly) and have been used to
demonstrate sperm nuclear chromatin remodelling and
nuclear envelope assembly. A comparable homologous
20   system for human and other mammalian sperm dispersion
and nuclear assembly has not been reported.

A method is described in WO95/21860 to transform the
human sperm to individual chromosomes using cell-free
25   extracts. The cells used in those studies were human
somatic cancer cells (HeLa). As discussed above, sperm
chromosomes are normally present in a highly condensed
state and one important aspect of preparing sperm for
fusion to other cells is to decondense the chromosomes.
30   This patent application proposed a method for
decondensation of sperm chromatin and/or chromosomes.
However this system has a number of serious
disadvantages. Firstly, the human sperm dispersion
(decondensation) was never uniform because, as discussed
35   below, the methods used to prepare sperm nuclei were
inadequate.

- 8 -

Secondly, the sperm dispersion in this system cannot be controlled, therefore, the chromosomes of the dispersed nucleus in the absence of a boundary (envelope) start breaking down within a short period of time. This is a serious problem in many applications which require the sperm to form a normal nucleus with intact chromosomes and rigid nuclear envelope (membrane).

Furthermore, it is highly desirable to be able to control the size of the transformed sperm nucleus (with envelope) as different sizes of transformed sperm nuclei can be used for various purposes. For example, a relatively large nucleus is required for fusion with an oocyte, a smaller nucleus for fusion with somatic cells and a very small nucleus for Intracytoplasmic sperm injection (ICSI). For practical purposes, it would be highly advantageous if transformed sperm nuclei, once assembled, could be stored in liquid Nitrogen (-176°C) or at -70°C. This is impossible with the methods described for sperm decondensation using HeLa cell-free extracts (WO 95/21860).

A protocol for in vitro mammalian sperm nuclear transformation has now been devised. For example, it has been shown that mature mouse or human sperm can be transformed into a nucleus capable of fusion with other cell types by incubating the sperm in cell free extracts from embryonic stem cells or developing embryos.

The present inventor has now devised a series of techniques for preparing sperm nuclei from spermatozoa and transforming the sperm nucleus morphologically to a form which can fuse with somatic cells, oocytes or be injected into oocytes.

First is described a technique for preparing sperm, more particularly for permeabilising sperm nuclei. Thus in a

- 9 -

first aspect, the present invention provides a method of
sperm cell permeabilisation which comprises,

(a)   washing a sample containing spermatozoa in a sperm
nuclear extraction buffer (SNEB) which includes one or
more metal chelating agents (e.g. EDTA/EGTA or
Bathophenanthroline sulphonate) and which is
substantially free of non-ionic detergents (such as
Triton X100) and divalent cations (such as Ca^{2+} or Mg^{2+}),

(b)   contacting the spermatozoa with one or more
permeabilising agents which create holes in the nuclear
membrane, such as lysolecithin (L-α-lysophosphotidyl-
choline) and/or digitonin, and

(c)   optionally separating the sperm tail from the sperm
head.

Prior to washing the spermatozoa in SNEB, the sperm
sample e.g. human semen sample is preferably treated to
separate active motile mature sperm for further steps,
this may conveniently be achieved by application of the
sample to a density gradient system such as a silica-
based density gradient. The gradient purified sperm are
preferably then washed in PBS or a similar buffer. If
used, the gradient and PBS or like buffer should be
substantially free of divalent agents as discussed
above. A sperm sample obtained directly from the
epididymis may conveniently first be filtered to remove
tissue debris.

The permeabilisation step (b) will conveniently be
carried out on ice for 10-30 minutes under regular mild
agitation. Lysolecithin is a preferred permeabilising
agent for use in step (b) and suitable concentrations
vary from 50-400, preferably 100-250 μg/ml, in a
particularly preferred embodiment spermatozoa are also
contacted with a further permeabilising agent such as
digitonin. Suitable concentrations for said further
permeabilising agent are 25-500, preferably 50-300 μg/ml

- 10 -

in the SNEB.

The SNEB will preferably contain 5-30 mM e.g. 15-25 mM
of HEPES-NaOH (pH 7.0), 50-300, eg 100-200 mM KCl, 100-
5   400, e.g. 200-300 mM sucrose and 1-100, preferably 1-20,
e.g. 3-10 mM of a chelating agent such as EDTA as well
as one or more protease inhibitors such as antipain,
aproteinin, leupeptin, chymostain and APMSF.

10   'Chelating' agents include molecules which are capable
of forming covalent (more particularly co-ordinate
bonds) with metal ions present in the sample or buffer.
The amount of chelating agent present is preferably
sufficient to sequester a significant proportion of the
15   available divalent cations and provide an environment
for sperm nucleus permeabilisation with a low
concentration of divalent cationic metal ions, typically
less than 200 μM, preferably less than 100 μM.

20   The permeabilisation step will preferably leave remnants
of the original sperm nuclear membranes associated with
the sperm nucleus i.e. holes are formed but parts of the
nuclear membrane remain intact and in situ. Triton X100
at 0.25% to 2.0% solutions are normally recommended for
25   human or animal cell nuclear isolations. However it has
surprisingly been found that Triton X100 is not suitable
for the methods of the present invention because it
causes too great a disruption in the nuclear membrane of
the sperm. For optimum sperm dispersion, discussed in
30   more detail below, it is important that factors in the
extracts used have access to the nuclear chromatin.
However, re-assembly of the new nuclear envelope after
dispersion has been found to be most efficient when
remnants of the original sperm nuclear membrane remain
35   associated with the dispersed nuclei. Thus,
permeabilisation agents are selected which are more
gentle and create holes in the nuclear membrane rather

- 11 -

than harsher reagents such as Triton. The SNEB in which
the sperm are preferably suspended when in contact with
the permeabilising agent(s) is substantially free of
Triton, in other words it is completely absent or
present at a concentration of no more than 0.1%,
preferably no more than 0.05%.

Similarly, it is usually recommended that the buffer for
nuclear preparation from human or non-human animal
somatic cells should contain some divalent cationic
agents, typically Ca^{2+}, Mg^{2+}, spermine or spermidine. It
has been found that incorporation of Ca^{2+}, Mg^{2+} or other
divalent cations in buffers used to isolate sperm nuclei
does not allow successful nuclear dispersion. They are
therefore omitted from PBS or any gradient system used
to purify sperm from semen or epididymis and from SNEB.
Functionally insignificant amounts of these cationic
species may be present, e.g. less than 50-100 micromolar
concentrations of divalent cations such as Ca^{2+} or Mg^{2+}.
However, we find that human sperm in the absence of low
concentrations of cationic agents may begin to aggregate
(clumping) following permeabilisation. Therefore, SNEB
for human sperm nuclear preparation preferably contains
0.1-1.0 mM, preferably 0.15-0.4 mM, e.g. 0.25 mM (each)
of spermine and spermidine in addition to e.g. 3-5 mM
chelating agents. Spermine and spermidine are used to
avoid clumping of the nuclei. For mouse sperm
preparation neither are necessary.

It has surprisingly been found that incorporation of
e.g. 2.5-20, preferably 5-10 mM chelating agents in the
SNEB dramatically improves the ability of the sperm
nuclei to disperse.

In the above method steps (a) and (b) may be separate or
simultaneous, e.g. the sperm sample may simply be
suspended in the SNEB to which one or more

- 12 -

permeabilising agents have already or may subsequently
be added.

A separate step for removal of the sperm tail is
5   generally not required for preparation of human sperm
nuclei. With human sperm, tail loss conveniently occurs
during the permeabilisation step but some sperm may
emerge from the preparatory (permeabilisation) method
with tails intact. Generally, the sperm will then lose
10   their tails during the incubations for dispersion/
reprogramming/reassembly.

In vivo transformation of the transcriptionally silent
sperm head to a male pronucleus consists of a series of
15   macromelecular events: sperm chromosome decondensation,
release of protamines, DNA repair, chromosomal
remodelling, assembly of organelles and a nuclear
envelope around the reprogrammed haploid chromosomes.
(Longo, F.J. Curr. Topics. Dev. Biol. 12: 149-184, 1978;
20   Collas, P and Poccia, D. Theriogenology, 49: 67-81,
1998). All these events are accomplished by molecular
chaperones, histones, non-histone structural proteins,
DNA-repair enzymes and factors transiently accumulated
in the egg ooplasm. Indeed, this maternal stockpile, in
25   amphibians and flies, is so extensive that the zygote
can replicate several thousand times independently of
parental gene activation.

In humans and higher mammals with placenta, successful
30   male pronuclear assembly and fertilisation are however
largely determined by the quality of the egg cytoplasm
(chromosome decondensation, DNA repair, demethylation
and remodelling factors). The inventors have discovered
that cell-free extracts incorporating all of these
35   factors can be prepared and used in cell-free systems to
transform sperm nuclei. Methods of preparing cell-free
extracts for transforming sperm (nuclei) into a form

- 13 -

capable of fusing with nuclei of other cells are
described herein. As a starting cell, any animal,
preferably mammalian cell can be used. While
differentiated somatic cells can be used, preferably the
cells from which the extracts are derived are
pleuripotent, more preferably ES cells or embryo cells.
The use of ES or embryo cells is particularly preferred
when the sperm nuclei is being transformed for fusion
with an oocyte or microinjection into egg cytoplasm (as
opposed to fusion with a somatic cell). The cells from
which the extracts are generated need not be from the
same species as the sperm which it is desired to
transform.

It has been found that cell-free extracts in which the
sperm (nuclei) can be incubated in order to achieve
dispersion and reprogramming prior to fusion are
preferably obtained from embryos or from embryonic stem
cells. The use of cell-free extracts (preferably
derived from embryos or embryonic stem cells) to
disperse, reprogramme and reassemble sperm nuclei
constitutes a further aspect of the present invention.

Thus, in a further aspect, the present invention
provides a method of dispersion of a sperm nucleus and
nuclear reprogramming and reassembly of the nuclear
envelope of said sperm nucleus, which method comprises
incubation of a sperm or sperm nucleus in one or more
cell-free extracts, preferably derived from embryos or
embryonic stem cells. The sperm/sperm nucleus is
preferably permeabilised prior to incubation in the cell
free extracts.

Herein, the preparation and use of cell-free extracts is
described with reference to ES cells or embryos but
extracts from other cell types may be prepared and used
in an analogous manner.

- 14 -

As discussed above, when incubation to achieve
dispersion/reprogramming/reassembly of the nuclear
envelope begins, some of the sperm nuclei may still have
their tails in tact, therefore reference herein to sperm
5   'nuclei' does not imply that the tail must be absent.

Mouse/human embryonic stem cells are pleuripotent
(undifferentiated) and can be grown using special
cultural conditions. The efficiency of the cell-free
10   extracts derived from developing mouse embryos at 11-12
days of gestation is comparable to that of ES cell-free
extracts.

A preferred incubation method comprises a two step
15   protocol wherein the nuclei are incubated in first one
then a second cell-free extract. Preferably at least
one, more preferably both of these extracts are obtained
from embryos or embryonic stem cells. Conveniently both
of these extracts are obtained from embryos
20   (conveniently 11-12 days post-coitus in mice or for
humans, during the first trimester) or embryonic stem
cells, preferably both extracts being from the same
batch of preparations.

25   Thus typically, the first extract is prepared from a
sample of embryos or ES cells. Typically, the embryos
will first be dissected into small fragments. The
samples are suspended in a buffer which is substantially
free of divalent cations, more particularly of Mg^{2+} and
30   Ca^{2+} and subjected to a gentle centrifugation step,
typically 1,000-5,000 rpm for 5 to 25 mins, preferably
1,500 to 2,500 rpm for 10 to 20 mins. The resulting
supernatant is referred to herein as extract A (or
dispersion extract) and is used in the first step of the
35   incubation methods described above. Extract A is
responsible for both sperm dispersion and nuclear
envelope assembly and thus must contain the materials

necessary for these processes. It is a novel and important feature of this extract that its components allow both dispersion and nuclear envelope assembly. While it could be envisaged that separate extracts or

5   components are prepared and contacted with the sperm, it is important for optimum performance of the present invention that dispersion and reassembly happen substantially at the same time. In this way, formation of the enlarged sperm pronucleus can be controlled and

10   breakdown of the chromatin avoided. Extract A thus provides a convenient mechanism for simultaneous dispersion and reassembly.

The pellet is resuspended in a buffer which may contain

15   divalent cations such as Mg^{2+} at concentrations of 2.5-10mM, preferably 5mM and 2-10% e.g. 5% glycerol. The resulting suspension is then subjected to a moderate centrifugation step e.g. spun at 8,000-20,000 rpm for 15-45 mins, preferably 10,000-15,000 rpm for 25 to 35

20   mins. The resulting supernatant provides the extract used in the second incubation step and is referred to herein as extract B (or reprogramming extract). This extract B provides the materials necessary for reprogramming of the sperm nuclear chromatin.

25

The second supernatant is preferably dialysed prior to use in the incubation method and particularly preferably undergoes a further centrifugation step after dialysing e.g. 3,000-6,000 rpm for 10 to 20 mins, e.g. 3,800-4,800

30   rpm for 12 to 17 mins. The pellet produced is discarded. The above described methods of extract preparation constitute further aspects of the invention.

As discussed above, extract A should be free of divalent

35   cations and extract B preferably contains Mg^{2+}. It may be possible to prepare extract B which does not contain Mg^{2+}, in which case, the two extracts may be brought

- 16 -

simultaneously or substantially simultaneously into
contact with the sperm, e.g. by prior mixing.

Thus in a preferred embodiment, the present invention
5   provides a method of sperm transformation which
comprises incubating a sample of sperm or sperm nuclei
(or a mixture thereof), in a dispersion extract and
subsequently incubating said sample in a reprogramming
extract. As discussed above, these extracts will
10   preferably be derived from embryos or embryonic stem
cells. Incubation in the dispersion extract preferably
lasts for between 15 mins and an hour and a half, more
preferably 30-60 mins and incubation in the
reprogramming extract preferably lasts for ½-2½ hours.
15

In a further preferred embodiment ATP (0.5-1 mM) and
creatine phosphate (5-10 mM) are added to the dispersion
extract before and/or after the sperm (nuclei) are added
for the first incubation step. The incubations will
20   preferably be carried out at 25-35°C e.g. around 30°C.

The sperm (nuclei) are preferably contacted with Mg^{2+},
ATP and creatine phosphate as well as Extract B at the
start of the second incubation.
25

GTP and glycogen are preferably added during the second
incubation step.

Extracts A and B and methods for their preparation
30   constitute further aspects of the invention. Preferred
components of the extracts are discussed but they are
most precisely described with reference to their methods
of preparation.

35   By "dispersion" of the sperm nuclei is meant
particularly decondensation of the nuclear chromatin as
described, for example, in WO95/21860. Preferably

- 17 -

during this step sperm-specific protamines may be
released and the initiation of the assembly of the new,
larger, nuclear envelope begins. Chromatin dispersion
and simultaneous initiation of nuclear envelope assembly
is preferably achieved according to the method of the
invention and this is particularly advantageous as it
limits chromosomal breakdown. No prior art systems have
achieved this for mammalian sperm.

The reaction of sperm nuclei to decondensation factors
in the egg is likely to be a factor in determining
fertility. Thus the dispersion and reprogramming
extracts have utility in the field of reproduction such
as fertility assessment, diagnosis of sub-fertility and
infertility treatment.

The ability of sperm nuclei to disperse and reassemble a
nuclear envelope are believed to be related to sperm
parameters such as viability and motility which are
indicative of their general health and functional
capacity. The spermatozoa incubated in IVF media prior
to ICSI progressively lost their ability to disperse and
assemble nuclei (see Fig. 3). Thus the quality of sperm
may be assessed on the basis of the response to
incubation in the extracts of the present invention.
Thus, in a further aspect the present invention provides
a method of testing sperm, in particular for its ability
to successfully fertilise an oocyte, which comprises
incubating a sample of sperm, particularly sperm nuclei,
in a dispersion and optionally a reprogramming extract
and measuring the degree of chromatin decondensation
and/or nuclear membrane assembly. Suitable techniques
for monitoring chromatin decondensation and nuclear
assembly, e.g. by visualisation using
chromatin/chromosome dyes or fluorescent assays or
immunoassays etc. are described in WO95/21860.

- 18 -

The reagents for use in such a diagnostic test may be
packaged as a kit. Thus, in a still further aspect, the
present invention provides a kit for testing sperm which
comprises a dispersion and optionally a reprogramming
5   extract as described above, optionally combined with one
or more reagents for use in the detection of decondensed
sperm chromatin and/or detection of a nuclear envelope.

Preferably, the dispersion results in enlargement of the
10   sperm nuclei (5-15 times increase in size) within 15-30
min of incubation. Sperm nuclei resistant to dispersion
fail to increase in size, and exhibit a distinctly
impermeable sperm membrane, even after several hours of
incubation (see Fig. 2).
15

During the second incubation step, the dispersed sperm
nucleus undergoes "nuclear reprogramming", which refers
to the process by which the sperm-specific chromosomal
proteins (protamines) are released and new oocyte-
20   specific (extract-specific) proteins are assimilated by
the sperm chromatin to form a novel nuclear structure
(also called chromatin remodelling). Additionally, the
haploid sperm DNA is extensively and selectively
modified by DNA methylases or demethylases present in
25   the egg ooplasm (or extracts) to alter the pattern of
methylation.

In a preferred embodiment magnesium ions, typically at
concentrations in the region of 5-10 mM, are added to
30   the reprogramming extract, optionally together with ATP
and creatine phosphate, e.g. at concentrations of 0.5-1
mM and 5-10 mM respectively.

Also during the second incubation step, the sperm
35   nucleus will preferably undergo complete or
substantially complete reassembly of the nuclear
envelope. Reassembly of the nuclear envelope begins

- 19 -

during the first incubation step. Preferably, the reprogramming of sperm nuclear chromatin and DNA repair takes place and glycogen (e.g. 0.5-3 mg/ml, such as 1 mg/ml) and GTP (preferably 0.5-1.0 mM) are added to

5   facilitate fusion of membrane vesicles and formation of a discrete nuclear envelope. The small and tightly packed sperm nucleus has been transformed by dispersion and then reprogramming into a much larger unit which after reassembly of the lipid bi-layer nuclear envelope

10   is morphologically much more similar to normal somatic cells or oocytes. Preferably the nuclear envelope will be at least 90% more preferably 95-100% complete after the second incubation step. This is determined by nuclear membrane-specific fluorescent staining or

15   reacting the nuclei with antibodies against lamins.

In a particularly preferred embodiment the second incubation step is itself made up of two parts, a reprogramming of chromatin and DNA repair step which

20   lasts for 15 to 60 mins and then a longer incubation period of 30 mins to 2 hours, typically 45 to 75 mins, after addition of GTP and glycogen during which the nuclear envelope forms. Although in alternative embodiments all components are added simultaneously and

25   incubation continuous until the nuclear envelope is formed.

The dispersion extract (extract A) preferably contains lipids, typically lamins and membrane vesicles, which

30   are utilised in the reassembly of the nuclear envelope. It is a particularly advantageous feature of the way in which the sperm nuclei are prepared in accordance with the method described above that fragments of the nuclear membrane remain and act as convenient nucleation points

35   for the build up of a complete or substantially complete nuclear membrane after dispersion. Thus preferably, it is the sperm nuclei prepared by the permeabilisation

- 20 -

method described herein which are then subject to the
above incubation/transformation techniques.

It is a particularly advantageous and novel feature of
5   the present invention that the sperm dispersion can be
controlled. Previous methods of dispersion led to a
rapid breakdown of the chromosomes of the dispersed
nucleus and thus the nucleus is not intact and available
for fusion with other cells. In contrast, according to
10   the methods of the present invention, the sperm nucleus
is transformed to a normal nucleus with a nuclear
envelope and is available intact for fusion with somatic
cells or oocytes. The methods of the invention allow
the size of the transformed sperm nucleus (with
15   envelope) to be controlled to generate sperm nuclei for
different purposes. The largest nuclei, diameter of
approx. 45-40 micrometer are used for fusion with an
oocyte, smaller nuclei having a diameter of approx. 20-
25 micrometer fuse better with somatic cells and very
20   small nuclei having a diameter of less than 20
micrometer are suitable for ICSI.

The eventual size of the transformed sperm nucleus can
be controlled by the length of time of incubation, the
25   longer the incubation period, the smaller the nucleus
(see Fig. 6). This reduction of nuclear size on
prolonged incubation is possibly due to the
recondensation of the reprogrammed sperm chromatin.
This transient condensation of the male pronuclear
30   chromatin precedes fusion with female pronucleus during
natural conception. We have observed that the reduction
in nuclear size reaches a minimum (approx. 15-20
micrometer diameter) and remains unchanged even after
several hours of additional incubation.
35

A further advantage is that the transformed sperm
nuclei, once assembled, can be stored in liquid nitrogen

- 21 -

(-176°C) or at -70°C for up to 4-6 months.

Thus, according to a further aspect of the present
invention is provided a transformed sperm nucleus whose
5   chromatin has undergone decondensation and reprogramming
and whose nuclear envelope has been reassembled to
provide a nucleus which is at least 5 times, preferably
at least 10-15 times larger than a normal untreated
sperm nucleus.
10

Decondensation of chromatin, nuclear (chromatin)
reprogramming and reassembly of the nuclear envelope are
defined above. "Transformation" refers to the whole
process by which a sperm nucleus undergoes extensive
15   biochemical and morphological alterations so that it is
capable of fusion with another cell and/or
intracytoplasmic injection into an oocyte.

In a further aspect, the methods described herein to
20   reprogramme sperm can also be used for reprogramming
somatic cells. Nuclear reprogramming is the epigenetic
transformation of a specific chromosomal organisation to
a state which leads to localised or global activation
and inactivation of genes. Global reprogramming is the
25   characteristics of male pronuclear assembly and of the
somatic cell nuclei transferred to oocytes observed in
fertilisation and in animal cloning studies,
respectively.

30   Nuclear reprogramming is the most critical step in
animal cloning studies. In current techniques, the
nucleus from the embryonic or adult cells is transferred
to the enucleated oocytes. The developmental potential
of the fully differentiated somatic cell nucleus is
35   exclusively determined by the cytoplasmic factors of the
oocyte and the competence of the chromosomes to undergo
remodelling in the transferred nucleus.

- 22 -

However, the egg cytoplasm often fails to achieve successful reprogramming and this is why current animal cloning is very inefficient. It is therefore proposed to overcome these problems by reprogramming the

5 differentiated somatic cell before it fuses with the enucleated oocyte. A particular advantage of reprogramming using the cell-free extracts described herein rather than relying on factors present in the oocyte cytoplasm is that while the amount of factors

10 present in a single cell is limited, the extracts can be prepared to ensure that the cell is exposed to all necessary factors at adequate concentrations.

Thus somatic cells may be permeabilised as described

15 herein, a preferred permeabilising agent being a mild detergent such as digitonin. An extract equivalent to the SNEB described herein is used but this typically contains a lower concentration of metal chelating agent e.g. <0.5 mM, preferably around 0.1 mM EDTA. The

20 permeabilised somatic cells are then treated with Extracts A and B as described herein. The somatic cell nucleus is already fairly large (much larger than a sperm nucleus) and the methods may therefore result in little or no increase in overall size of the nucleus.

25 However nuclear reprogramming takes place. Following reprogramming, the cells can conveniently be separated from the extracts by layering over (1-3 mL) of (1.1M) sucrose in SNEB and then centrifuged at 2.5K for 30 mins. The cells will settle at the bottom while extract

30 proteins will remain at the top. These cells can then be used for fusion with enucleated oocytes according to known animal cloning techniques.

To assess the extent of remodelling/reprogramming,

35 methylation of DNA and histones can be monitored by incorporating S-adenosyl methionine in the extract(s).

- 23 -

Two kinds of methylation changes would be expected:
Global alteration in methylation of DNA (centromere and
heterochromatin) and histones or localised effect on
imprinted genes. Imprinted genes are marked by
5   methylation in the gamete and can be erased only by
germline transmission. Correct reprogramming in vitro
would mean that methylation imprints passed on to the
somatic cells by gametes (sperm or egg) would remain
intact while methytation of other genes altered. These
10   changes can be determined by standard molecular and
biochemical methods.

Having prepared the sperm nuclei which are stable and
transformed and have an intact nuclear envelope, there
15   are many uses of the nuclei which have exciting
therapeutic, diagnostic and scientific implications.
Technologies have been developed to fuse two different
normal (somatic) cells but these are not applicable to
sperm-egg fusion because the human sperm are tiny,
20   highly specialised and condensed cells. The transformed
sperm nuclei of the invention are capable, through the
morphological changes which they have undergone *in
vitro*, to fuse with an oocyte or somatic cell.

25   Thus a preferred use of the transformed nuclei of the
invention is in fusion with somatic cells or oocytes.
In a further aspect, therefore, the present invention
provides a method of fusion, e.g. chemical, biochemical
or electrical fusion, electrical fusion (electrofusion)
30   being preferred, between a transformed sperm nucleus and
a somatic cell or oocyte. For the first time is
provided a method of *in vitro* fusion between a sperm
nucleus and the nucleus of another cell and thus a new
method of *in vitro* fertilisation.
35

Electrofusion between the transformed sperm nucleus and
an egg (oocyte) has many advantages over ICSI, the

- 24 -

conventional method for *in vitro* fertilisation for male
factor infertility. It is simple, efficient (80-90%)
and does not rely on operator ability; it is also much
quicker many oocytes and sperm being fused in minutes.
Mechanical damage to the oocyte by this method is
negligible. Formation of the male pronucleus and
electrical activation ensures subsequent formation of
the female pronucleus which does not routinely happen in
ICSI.

Standard electrofusion techniques may be used Collas,
P., Fissore, R. and Robl, J.M., Anal. Biochem. 208: 1-9.
1993; Willadsen, S.M. Nature 320: 63-65, 1986; Bertsche,
U. and Zimmermann, U. Radiat. Enviorn. Biophys. 27: 201-
212, 1988; Lee, J.Y *et al.*, Nat. Genet. &: 29-33, 1994.
Preferably, the sperm nuclei are pre-aligned on an
electrode by a brief electrical pulse prior to mixing
with eggs and electrofusion. Cell-tracking markers or
specific genetic markers can be used to confirm a
successful fusion as described in the Examples. A
typical protocol comprises addition of a cell-tracking
marker 15 mins before the end of the reprogramming/
reassembly incubation step. The nuclei are isolated by
sucrose velocity gradient sedimentation and may be
counted in a hemocytometer. The sperm nuclei can also
be isolated individually by micromanipulation. The
sperm nuclei and eggs are mixed at a ratio of 2:1 in a
volume of approximately 60-80µl (Collas,P., Fissore, R.
and Robl, J.M., Anal. Biochem. 208: 1-9. 1993).

Electrofusion can then be performed in an Electrocell
Manipulator 200/Microslide (Genetronics, Inc. BTX
Instrument Division, San Diego, California) with 1mm
electrode gap. The embryo may then be cultured and
optionally prepared for implantation so that it may
proceed to term *in vivo*. The embryo may be cultured to
blastocyst *in vivo* or *in vitro* and it may be desirable

- 25 -

to screen at the blastocyst stage for suitability for development to term, such methods are described, *inter alia*, in GB-A-2331751.

5   Mixing sperm and egg at a ratio of around 2:1 is particularly preferred in order to maximise the proportion of single sperm:egg fusion events, as opposed to sperm:sperm fusion, multiple sperm:egg fusion or egg:egg fusion. Using cuvettes with a capacity of 40-
10   80μl and 1mm electrode gap, the sperm nuclei can be pre-aligned on the electrode by a brief electrical pulse prior to mixing with eggs and electrofusion. Single cells can be placed in BTX Microslide Cuvettes which can be fitted to a microscope with standard optics to
15   provide the additional benefit of allowing visual alignment of a single egg and sperm nucleus between 0.5 mm stainless steel electrodes prior to electrical pulsing.

20   Methods of egg (oocyte) isolation from various animals are well-known in the art and any suitable technique for harvesting human or non-human oocytes may be used to provide oocytes for use in the fusion techniques of the present invention.
25

A further aspect of the present invention comprises an embryo prepared by *in vitro* fusion between a sperm nucleus and an oocyte. Typically the sperm nucleus will be a transformed cell nucleus prepared by one of the
30   methods described herein. As discussed above, the present invention provides opportunities for IVF of great benefit to couples who are experiencing difficulties with conception. The invention is also of use in the preparation of non-human embryos, referred to
35   herein as animal embryos which may be of particular use in agricultural contexts.

- 26 -

According to a further aspect of the invention is
provided a method for preparing an animal, which method
comprises preparing an animal embryo as described above,
i.e. by *in vitro* fusion between a sperm nucleus and an
5   oocyte and causing the animal to develop to term from
said embryo and optionally breeding from the animal so
formed. These latter stages may be performed as
described in GB 2331751.

10   A further application of the present technology is in
sperm-mediated gene transfer (transgenics). In this
case the sperm nuclei can be pre-incubated with
exogenous genes in a suitable vector prior to sperm
dispersion.
15

Fusion of the sperm nuclei with oocytes has been
discussed in some detail above. A further application
of the present technology is in fusion with somatic
cells in the diagnosis of sperm chromosome
20   abnormalities. At present there are few techniques
available for genetic analysis of sperm because of the
dense packing of chromatin in the sperm heads.

In a further aspect, the present invention provides a
25   method of testing for sperm chromosome abnormalities,
which method comprises contacting a transformed sperm
nucleus whose chromatin has undergone decondensation and
reprogramming and whose nuclear envelope has been
reassembled with a somatic cell and causing the two
30   nuclei to undergo fusion (e.g. electrofusion) and
subsequent cytogenetic and/or fluorescent in-situ
hybridisation (FISH) analysis of the chromosomes to
identify abnormalities. (Martini E. *et al.*, Hum.
Reprod. 12: 2011-2018, 1997; Vegetti, W. et al., Hum.
35   Reprod. 15: 351-365, 2000).

Suitable somatic cell types include Indian Female

- 27 -

Muntjac fibroblast cells or ES cells, cultured somatic
cells generally being preferred. The chromosomes may
conveniently be investigated by cytogenetic analysis
using stains and paints.

5

Use of a transformed nucleus as described above in a
method of testing for sperm chromosome abnormalities,
particularly methods which involve electrofusion of said
nucleus to another cell or cells constitutes a preferred
10   aspect of the present invention. Diagnostic methods are
conveniently performed using a kit and in a further
aspect the present invention provides a kit for testing
for sperm chromosome abnormalities which comprises
Extract A and preferably Extract B as defined above,
15   optionally together with a somatic cell capable of
fusion with a sperm nucleus e.g. Indian female muntjac
cells and optionally together with reagents for
permeabilisation of sperm nuclei. Such agents, and an
SNEB which may also be included are described above.

20

Reagents suitable for such isolation and transformation
methods are described above. Means for chromosome
analysis include culturing the fused cells in the
presence of a selectable marker (drugs) and generating
25   the metaphase chromosomes by temporary incubation in the
presence of colcemid. The drug would allow the growth of
only hybrid cells and colcemid would facilitate
producing visible metaphase chromosomes.

30   As well as fusion between a transformed sperm nucleus as
described herein and the nucleus of an oocyte or somatic
cell, the transformed sperm nucleus may be injected into
the cytoplasm of an egg in a manner analogous to ICSI.
Thus in a further aspect the present invention provides
35   a method of in vitro fertilisation which comprises
microinjection of a transformed sperm nucleus as
described herein (i.e. obtainable or obtained by the

- 28 -

processes described herein) into the cytoplasm of an oocyte. This technique has advantages over conventional ICSI techniques in that it avoids the need for oocyte induced reprogramming.

5

The invention will now be further described in the following non-limiting Examples with reference to the accompanying Figures in which:

10   Fig. 1a shows the experimental steps used to disperse sperm nuclei in Extract A which also provides membrane vesicles to initiate nuclear envelope assembly during the first hour of incubation. Addition of ATP and creatine phosphate (CP) at this step enhances sperm

15   dispersion. Extract B, Mg^{2+}, additional ATP and CP allow chromatin remodelling and nuclear reprogramming to take place. GTP and glycogen aid fusion of the assembled vesicles to form a complete nuclear envelope.

20   Fig. 1b shows the various steps involved in sperm-egg fusion, sperm-mediated transgenics and methods of diagnosis of human sperm chromosome abnormalities.

Fig. 2 shows that Mg^{2+} added to the buffers during sperm

25   nuclear isolation or added exogenously to the extracts, severely inhibits sperm nuclear dispersion. It is for this reason, that at the initial phase of incubation (Fig. 1a) Mg^{2+} is omitted. Phase contrast micrographs show the general morphology of the sperm under a light

30   microscope, whereas, DAPI and HDCC highlight the DNA and nuclear envelope, respectively, under fluorescent microscope.

Fig. 3 demonstrates that continued incubation of

35   gradient purified human spermatozoa in IVF medium (Medicult) inhibits sperm dispersion. The staining of the spermatozoa with lipophilic fluorescent stain HDCC

- 29 -

suggests that increased stability of the sperm nuclear
membrane on prolonged incubation could be responsible
for the resistance of the sperm to dispersion. This
figure also shows that addition of ATP and CP during
5   initial phase of incubation in Extract A (Fig. 1a)
enhanced sperm dispersion.

Fig. 4 shows mouse sperm nuclear dispersion, chromatin
remodelling and very early stage of re-assembly of the
10   nuclear envelope. The steps used are shown in Fig. 1a.
Very early phase of sperm dispersion is shown in D, E
and F (compare with controls A-C). Membrane vesicles
are already associated with the dispersed sperm (F) in
the first hour of incubation. When extract B, Mg^{2+} and
15   CP were added, the chromosomes of the dispersed nuclei
underwent reprogramming and the nuclear envelope became
more defined (G to L).

Fig. 5 shows continued incubation of remodelled nuclei
20   in the presence of GTP and glycogen facilitates a)
fusion of the vesicles to produce a distinct nuclear
envelope and b) reduction in the size of the nucleus.
The envelope assembled around remodelled nuclei (Fig. 4)
became distinct (A and B) and the nuclei became smaller
25   on further incubation (compare a & b with c and d in A).
These nuclei can be stored at -70°C without changing the
morphology on thawing.

Fig. 6 demonstrates transformation of human sperm nuclei
30   at various stages as shown for mouse sperm in Figs. 4 &
5. The sperm nuclei dispersed within 1 hour of
incubation (A-C), gradually became more dense and
smaller on further incubation (D to L). Occasionally,
some sperm nuclei (but less than 1%) failed to undergo
35   transformation even after hours of incubation as shown
in J, K and L.

- 30 -

Fig. 7 comprises photographs showing the relative size and morphology of human sperm, remodelled (i.e. reprogrammed) sperm and muntjuc cells.

5   Fig. 8 comprises photographs showing the chromosome composition of muntjac and hybrid cells which were selected for drug resistance after fusion with human sperm nuclei.

10   Fig. 9 comprises photos of hybrid cells and human lymphocytes which indicate the presence of human chromosomes in the muntjac hybrid cells.

15   **EXAMPLES**

Reagents

All standard chemicals (Dithiothreotol, DTT, Potassium chloride, Potassium acetate; Magnesium chloride and
20   Magnesium acetate, Sodium chloride, Sucrose, Glycerol, ethanol etc) were molecular biology grade from BDH. Nucleotides (ATP and GTP), Percoll (Molecular biology grade) were from Amersham-Pharmacia. Micrococcal nuclease (MiNase) and Deoxyribonuclease I (DNase I),
25   Restriction enzymes, glycogen and proteinase K, protease inhibitor cocktails were from Promega/Amersham-Pharmacia or Boehringer & Manheim. Lysolecithin, Digitonin, Dimethylsulphoxide, Creatine Phosphate, Propidium Iodide, DAPI, Vectachield and reagents for recombinant
30   DNA work were from Sigma Chemicals. HDCC, a lipophilic fluorescent membrane stain was from Calbiochem. Cell culture media, antibiotics, cell freezing media, trypsin-EDTA, colcemid were from Life Technologies. Dialysis tubings (Mr cut of 3, 500) were from
35   Spectraphor.

- 31 -

Preparation of Mouse embryo and embryonic stem (ES)
cell-free extracts: Extracts A and B

Developing mouse embryos were obtained from female mice
5   (C57 BL/6) at 11th or 12th day of pregnancy. The
placenta and other extra embryonic tissues were
surgically removed under low-magnification microscope
and the embryos were transferred to ice-cold PBS in a
sterile beaker held on ice. Henceforth, all the
10   operations were carried out on ice or at 4°C.

The embryos were dissected into small fragments and were
extensively washed with ice-cold PBS The tissue sections
were transferred to a loose-fitting glass homogenizer
15   and were further reduced to smaller sizes (6-8 strokes).
The material was spun at 1200 rpm for 5 min. and the
supernatant was discarded. The embryonic tissues were
resuspended in 20 mM HEPES-NaOH, pH 8.0, 10 mins KCl,
0.5 mM DTT and 1X protease inhibitors cocktail and held
20   on ice for 10 mins.

The material was transferred to a tight-fitting Teflon-
coated glass homogenizer and homogenized on ice with 8-
10 strokes. The homogenate was transferred to sterile
25   plastic tubes and spun at 2,000 rpm for 15 mins. The
supernatant (S) was carefully removed to another fresh
tube and the pellet (P) containing the rest of the
tissues was held on ice. The KCl concentration in S-
fraction was adjusted to 100 mM, centrifuged at 2,500
30   rpm prior to dialysis and designated as Extract A. The
P-fraction was resuspended in a buffer containing 20 mM
HEPES, pH 8.0, 0.2 mM EDTA, 0.5 mM DTT, 5% glycerol and
1X protease inhibitors cocktail prior to adding 0.6 M
NaCl in small drops. The suspension was gently mixed
35   for 15 min and spun at 13,000 rpm for 30 min.

The supernatant was carefully removed (without

- 32 -

disturbing the pellet) and additional glycerol was added
to achieve final concentration to 20%. This material
after dialysis was called Extract B. The dialysis was
carried out in 400-500 volumes of a buffer (20 mM HEPES-
NaOH, pH 8.0, 100 mM KCl, 0.2 mM EDTA, 0.5 mM DTT and
20% glycerol) for 6 h with one change. The dialysis bag
was from Spectraphor with a molecular weight cut off of
3,500. Dialysis tubes with higher molecular weight cut
off increases the precipitation of proteins resulting in
poorer quality extracts. Following dialysis, the
extracts were further centrifuged at 4,300 rpm for 15 mm
and the supernatant was aliquoted, snap frozen in liquid
Nitrogen and stored for at least 6 months at -70°C
without losing activities.

Mouse embryonic stem cells (EFC-1, passage 16, obtained
from Western General Hospital, Edinburgh were plated on
mitomycin-treated feeder layers (STO) in 90-mm pre-
gelatinised plastic dishes and cells were routinely
maintained undifferentiated by growing in the presence
of LIF (leukocyte inhibitory factor). In order to
obtain large number of cells, the cells were subcultured
by plating without feeder layer (STO) for 2 h and
subsequently transferring the media containing
unattached ES cells to fresh gelatin-coated dishes.
This procedure removes the residual feeder cells. The
ES cell colonies were detached from the plates using
rubber-policeman in cold PBS and were used to prepare
cell-free extracts exactly as described for mouse
embryos.

Generally, none of our buffers contain Mg^{2+} or other
divalent cations. Conventionally, the cell-free
extracts reported for studying transcription, DNA
replication or repair *in vitro* contain varying
concentrations of Mg^{2+}. As we shall discuss below, Mg^{2+}
is required for nuclear remodelling and certain steps of

- 33 -

nuclear envelope assembly in our system. Therefore, we add magnesium exogenously at a specific time point during incubation. It must be emphasized however that cationic interference is only prominent at the initial

5   phase of incubation during sperm dispersion. Once the sperm dispersion is complete, Mg^{2+} has no effect. On the contrary, this divalent cation helps the rest of the processes during incubation. Based on this logic, we have also prepared Extract B (but not extract A) in

10   buffers containing 5 mM Mg^{+2} and they work well.

Unlike reported cell-free extracts, we find that high speed centrifugation (60-70, 000 rpm/>200,000g in an ultracentrifuge) of extracts (more specifically Extract

15   A) significantly reduced the sperm dispersion activity and dramatically affected the nuclear envelope assembly.

Preparation of mouse and human sperm nuclei

20   The whole epididymis and vas deferens were recovered from 42-48 sexually mature (50-70 day old) C57BL/6 mice in a Petridish containing cold phosphate buffered saline without Ca^{2+} and Mg^{2+}. The epididymis were opened by sharp incisions with a razor blade and the mature

25   spermatozoa were allowed to swim out by gently agitating the dish for 10-15 mm at room temperature. The spermatozoa released in the buffer were filtered through 8 layers of gauze to remove the tissue debris. The filtrate was spun at 1200 rpm for 15 mm at 4°C.

30

The pellet was washed 2-3 times in sperm nuclear extraction buffer (SNEB: 20 mM HEPES-NaOH, pH 7.0, 150 mM KCl, 250 mM sucrose, 10 mM EDTA and 1X protease inhibitors cocktail (antipain, aproteinin, leupeptin,

35   chymostain and APMSF) and was resuspended in the same buffer. Mature spermatozoa were separated from less differentiated spermatocytes by sedimenting through 40%

- 34 -

percoll gradients equilibrated with SNEB. The gradient
purified spermatozoa were resuspended in SNEB containing
1-α-phosphotidyl choline (lysolecithin, Sigma), 10 mM
DTT and sonicated for 30 seconds (10 seconds each time).
5   It has also been found that the lysolecithin
concentration could be reduced 10-fold by adding
digitonin (100-200 µg/ml) in the SNEB during
permeabilisation. The spermatozoa were permeabilised on
ice for 15 min (regular agitation) and the reaction was
10   stopped by adding 3 volumes of SNEB containing 3 mg/ml
bovine serum albumin (BSA).

In order to separate tails from the sperm head, the
pellet resuspended in a small volume was layered over
15   1.1M sucrose cushion and was sedimented by
centrifugation at 4500 rpm for 30 min at 4°C. The
nuclei recovered following centrifugation were washed
with SNEB containing 10 mM DTT. The number of nuclei/ml
was determined by counting several dilutions in a
20   haemocytometer and the preparation was stored in SNEB
containing 50% glycerol at -70°C.

Preparation of human sperm nuclei

25   Human semen samples were obtained from healthy donors at
the St. George's Assisted Conception Unit (ACU). The
semen was liquefied at 37°C for 30 min prior to
separation of the motile mature spermatozoa by
discontinuous silica-based density gradients
30   (recommended for human sperm preparation). The gradient
purified spermatozoa were washed in PBS and the nuclei
were prepared as described above with the following
exceptions: The brief sonication step, final
sedimentation by layering the permeabilised nuclei over
35   1.1 M sucrose cushion were omitted and EDTA
concentration in SNEB was reduced to 3-5 mM and 0.25 mM
(each) of spermine and spermidine added to avoid

- 35 -

clumping of the nuclei (Spermine and spermidine were not used for mouse sperm nuclear preparation). The brief sonication step was avoided because unlike mouse sperm, the tails of the human sperm usually dissociate from the head during permeabilisation and/or incubation in extracts. After permeabilisation and transformation typically 90-95% of human sperm tails are lost.

Sperm dispersion, nuclear reprogramming and re-assembly of nuclear envelope in cell-free extracts (embyonic or embryonic stem, ES cells).

A general outline of the procedures are presented in Fig. 1a. 2-3 x 10^5 lysolecthin plus digitonin permeabilised sperm nuclei (human or mouse) were resupended in 50 μl buffer (20 mM HEPES-NaOH, pH 8.0, 150 mM KCl) containing 0.5 mM DTT, 0.5 mM ATP, 5-10 mM creatine phosphate, 1X protease inhibitors cocktail and 10-20 μl Extract A (70-100 μg of total protein) and the materials in the tube were gently mixed on ice prior to incubation at 30°C. After 30 min of incubation, a 2 μl aliquot was mixed with fluorescent dyes reconstituted in above buffer (DAPI plus HDCC or PI alone) on a microscope slide and was examined in an epifluorescent microscope (Nikon, Eclipse, E 400) connected to a system for image processing and analysis (LUCIA, version 4.21). When >90-95% of the nuclei dispersed (takes between 30-60 min), the tube was transferred to room temperature and 15-20 μl Extract B (225-300 μg of total protein) was added, and the ATP, CP and Mg^{2+} concentrations were adjusted to 0.5 mM, 5-10 mM and 5-6 mM respectively, in a 100 μl total volume. Following 30 min of further incubation, GTP and Glycogen were added to 0.5-1.0 mM and 1-2 mg/ml respectively. The envelope assembly was examined and the images were saved. The nuclei with re-assembled nuclear envelope can be stored at -70°C. The nuclear reprogramming was examined by molecular methods.

- 36 -

Isolation and culture of mouse oocytes

For immature oocytes, B6CBF1 (21-24 days) or MF1 female
mice (21 days) are injected with 7.5 IU eCG. The ovaries

5   are removed 48-52h post-injection into M2 medium (Sigma)
containing 10% fetal bovine serum (ICN Flow) and 0.25 mM
dibutyryl cyclic AMP (db CAMP, Sigma). The oocytes
released by puncturing large antral follicles with a 25G
hypodermic needle are denuded by repeated pipetting *in*

10   the presence of a low concentration of hyaluronidase
(Sigma). To obtain mature oocytes, the animals are
injected with eCG 48-52 h prior to second injection with
5IU of hCG. The cumulus masses recovered by squeezing
the oviduct 13.5-16 h post hCG in M2 medium containing

15   fetal bovine serum and the mature oocytes denuded by
hyaluronidase treatment are maintained in M2 media prior
to fusion.

Embryonic stem cell and Indian female muntjac fibroblast
20   culture:

These may be prepared by standard methods as described,
for example, in Sen, P and Sharma, T. Cytogenet. Cell
Genet. 39: 145-149, 1985; Lee, J.Y. *et al.*, Nat. Genet.

25   7: 29-33, 1994.

Electrofusion and culture of the embryo:

A cell-tracking fluorescent probe chloromethyl

30   derivative of fluorescin diacetate (CMFDA), which forms
an adduct with intracellular thiols and is stable for
several generations, is added during incubation of sperm
nuclei in extracts (Fig. 1). The cell-tracking marker
is incorporated into sperm during nuclear remodelling in

35   the cell-free systems. The marker is colourless and
nonfluorescent until cytosolic esterases cleave off the
acetate, releasing a brightly green fluorescent product

- 37 -

The sperm nucleus fused to an egg activates the
fluorophor to produce a green fluorescence (at visible
wavelength) of viable embryos resulting from sperm-egg
fusion. Alternatively, a specific genetic marker (e.g.
5   neo-lacZ vector) is pre-incubated with sperm nuclei
prior to nuclear transformation in cell-free extracts
and expression of these proteins (drug resistance and
staining) in the embryo or fused cells confirms
successful fusion and transgenesis.

10

The efficiency of fusion technology has improved
dramatically (80-90%) in the past 2-3 years. This is
primarily due to improved alignment of cells, better
design of fusion chambers and cell-tracker monitoring
15   systems. Microslide cuvettes (BTX) with a capacity of
40-80 μl and 1 mm electrode gap may be used. In these
cuvettes the sperm nuclei could be pre-aligned on the
electrode by a brief electrical pulse (AC, pearl string)
prior to mixing with eggs and electrofusion (DC).

20

Suitable electrofusion techniques are known in the art
and described, for example, in Takahashi, M. et al.,
Leukemia Research 15: 507-513, 1991; Nishi, T. et al.,
Cancer Research 56: 1050-1055, 1996; Meng, J. et al.,
25   Biol. Reprod. 57: 454-459, 1997; Saunders, J. Guide to
electroporation and Electrofusion, 227-247, 1991; BTX
Protocols PR0182, PR0336, PR0342, 1998; Miramatsu et
al., Int. J. Mol. Med. 1: 55-62, 1998.

30   **Embryo culture and uterine transfer**

Standard methods are suitable and described, for
example, in Gordon, J.W. Metods Enzymol. 225: 747-771,
1993; Kimura, Y. and Yanagimachi, R. Biol. Reprod. 52:
35   709-720, 1995; Lacham-Kaplan, O. and Trounson, A. Hum.
Reprod. 10: 2642-2649, 1995; Wakayama, T. et al., Nature
349: 369-374, 1998; Perry, A.C. et al., Science 284:

- 38 -

180-183, 1999.

Sperm-somatic cell fusion

5   The Human sperm nuclei were reprogrammed into somatic-
       type nuclei in mouse embryonic cell-free extracts as
       described herein.

       The relative size and morphology of the *in vitro*
10   reprogrammed human sperm nuclei and muntjac cells are
       shown in Fig. 7. Muntjac cells were grown to
       semiconfluence (12-16h) in 90 mm Petri dishes. The
       cells were washed three times with serum-free media
       prior to treatment with phytohaemaglutinin (100-200
15   μg/ml) resuspended in the same media. After 1h
       incubation at 37°C, the excess phytohaemoaglutinin was
       removed and sperm nuclei remodelled in the presence of
       Neo^r plasmid marker were added. Following incubation for
       2h, the excess unattached sperm nuclei were removed and
20   fusion was carried out for 1 mm in the presence of 50%
       poleethyleneglycol (PEG). The PEG was removed by
       washing the plates at least 4-5 times with Ca^{+2}/Mg^{+2} free
       PBS. The fused cells were maintained in complete media
       for 48h. The drug-resistant clones were selected for 3-
25   4 wks in the presence of 500-1000 μg/ml of G418. The
       drug resistant clones were picked individually and were
       expanded in complete media without drug by sequential
       transfer to multi-well plates. The cells were treated
       with 0.5 μg/ml colcemide for 6h to examine the metaphase
30   chromosomes of the fused cells (Fig. 8). The presence
       of human chromosome was determined by fluorescent in
       situ hybridisation (FISH) using human pan-centromere
       probe. As a control, the human lumphocyte metaphase
       chromosomes were hybridised under identical conditions
35   (Fig. 9).

- 39 -

Claims

1.   A method of sperm permeabilisation comprising:

5   (a)   washing a sample containing sperm in a sperm
           nuclear extraction buffer (SNEB) which includes one
           or more metal chelating agents and which is
           substantially free of non-ionic detergents and
           divalent cations;

10   (b)   contacting the sperm with one or more
           permeabilising agents; and

     (c)   optionally separating the sperm tail from the sperm
15   head.

2.   A method as claimed in claim 1 wherein the metal
chelating agent is selected from the group comprising
EDTA, EGTA and Bathophenanthroline sulphonate.
20

3.   A method as claimed in claim 1 or claim 2 wherein
the permeabilising agent is lysolecithin or digitonin.

4.   A method as claimed in any preceding claim wherein
25   the SNEB contains one or more protease inhibitors.

5.   A method as claimed in any preceding claim wherein
after contact with a permeabilising agent, parts of the
nuclear membrane remain intact.
30

6.   A method as claimed in any preceding claim wherein
the SNEB contains spermine and/or spermidine.

7.   A method of sperm transformation which method
35   comprises incubating a sample of sperm or sperm nuclei
in one or more cell-free extracts.

- 40 -

8.   A method as claimed in claim 7 wherein the cell-
free extracts are derived from embryos or embryonic stem
cells.

5   9.   A method as claimed in claim 7 or claim 8 wherein
the sperm or sperm nucleus is permeabilised prior to
incubation in the cell-free extract.

10.   A method as claimed in any one of claims 7 to 9
10   wherein said sperm or sperm nuclei are incubated in a
cell-free dispersion extract and in a cell-free
reprogramming extract.

11.   A method as claimed in claim 10 wherein said
15   dispersion extract contains the necessary components for
reassembly of a nuclear envelope.

12.   A method as claimed in claim 10 or 11 wherein the
dispersion extract comprises a supernatant generated by
20   centrifugation of a cell sample.

13.   A method as claimed in claim 12 wherein said cell
sample is a sample of embryos or embryonic stem cells.

25   14.   A method as claimed in claim 12 or claim 13 wherein
the reprogramming extract comprises a supernatant
generated following resuspension and centrifugation of
the pellet formed on generation of the supernatant used
in the dispersion extract.
30
15.   A method as claimed in claim 14 wherein the
reprogramming extract contains magnesium ions.

16.   A method as claimed in claim 14 or 15 wherein the
35   supernatant used in the reprogramming extract undergoes
a further centrifugation step.

- 41 -

17. A method as claimed in any one of claims 10 to 16
wherein the sperm or sperm nuclei are incubated in said
dispersion extract for between 15 and 90 mins and in
said reprogramming extract for between 30 and 150 mins.

18. A method as claimed in any one of claims 10 to 16
wherein said dispersion extract contains ATP and
creatine phosphate.

19. A method of preparing a sperm dispersion extract
from a cell sample which comprises:

(a)   suspending the sample in a buffer which is
       substantially free of divalent cations;

(b)   centrifuging the sample at 1,000-5,000 rpm for 5 to
       25 minutes; and

(c)   collecting the supernatant product of step (b).

20. A method of preparing a sperm reprogramming extract
which comprises:

(a)   suspending the pellet product of step (b) of claim
       19 in a buffer which optionally contains magnesium
       ions;

(b)   centrifuging the suspended pellet of step (a) at
       8,000-20,000 rpm for 15-45 mins; and

(c)   collecting the supernatant product of step (b).

21. A method as claimed in claim 19 or 20 wherein the
cell sample is an embryo or embryonic stem cell sample.

22. A method as claimed in claim 17 wherein the
incubation in said reprogramming extract is in two

- 42 -

parts, a first part which lasts for 15 to 60 minutes and
a second part which lasts for 30 to 120 minutes and
takes place in the presence of GTP and glycogen.

5   23. A method of testing a sample of sperm or sperm
nuclei which comprises:

       (a) incubating the sample in a cell-free dispersion
            extract as defined in any one of claims 11 to 21;

10
       (b) optionally further incubating the sample in cell-
            free reprogramming extract as defined in any one of
            claims 13 to 21; and

15   (c) measuring the degree of chromatin decondensation
            and/or nuclear membrane assembly.

       24. A kit for use in a method as claimed in claim 23
       which comprises:

20
       (a) a cell-free dispersion extract as defined in any
            one of claims 11 to 21;

       (b) optionally a reprogramming extract as defined in
25   any one of claims 13 to 21; and

       (c) optionally one or more reagents for the detection
            of decondensed sperm chromatin and/or detection of
            a nuclear envelope.

30
       25. A method or kit as claimed in any one of claims 10
       to 24 wherein said dispersion extract contains lipids.

       26. A method or kit as claimed in claim 25 wherein said
35   dispersion extract contains lamins and/or membrane
       vesicles.

- 43 -

27. A transformed sperm nucleus obtainable by the method of any one of claims 7 to 18.

28. A transformed sperm nucleus whose chromatin has undergone decondensation and reprogramming and whose nuclear envelope has been reassembled to provide a nucleus which has a diameter of 15 to 50 micrometers.

29. A method of *in vitro* fusion between a transformed sperm nucleus and a somatic cell or oocyte.

30. A method as claimed in claim 29 wherein the transformed sperm nucleus is as claimed in claim 27 or claim 28.

31. A method of electrofusion between a transformed sperm nucleus as claimed in claim 27 or claim 28 and an oocyte.

32. A method as claimed in claim 31 wherein the sperm nuclei and oocytes are mixed at a ratio of 1:1 to 3:1.

33. A method of testing for sperm chromosome abnormalities, which method comprises contacting a transformed sperm nucleus as claimed in claim 27 or claim 28 with a somatic cell and causing the two nuclei to undergo fusion and subsequent analysis of the chromosomes.

34. A method as claimed in claim 33 wherein the chromosomes are analysed by cytogenic or fluorescent in-situ hybridisation analysis.

35. A kit for use in a method as claimed in claim 33 or 34 which comprises:

- 44 -

(a)   a cell-free dispersion extract as defined in any
      one of claims 11 to 21;

(b)   optionally a reprogramming extract as defined in
      any one of claims 13 to 21; and

(c)   optionally a sample of somatic cells capable of
      fusion with a sperm nucleus.

36. A method of *in vitro* fertilisation which comprises
microinjection of a transfused sperm nucleus as claimed
in claim 27 or claim 28 into the cytoplasm of an oocyte.

37. A method of reprogramming a somatic cell which
method comprises incubating said somatic cell in one or
more cell free extracts.

38. A method as claimed in claim 37 wherein the cell-
free extracts are derived from embryos or embryonic stem
cells.

39. A method as claimed in claim 37 or claim 38 wherein
said somatic cell is incubated in a cell-free dispersion
extract and in a cell-free reprogramming extract as
defined in any one of claims 11 to 18.

40. An animal embryo prepared by transferring the
reprogrammed nucleus of a diploid donor cell i nto a
suitable recipient cell.

41. An embryo as claimed in claim 40 wherein the
recipient cell is an oocyte.

42. An embryo as claimed in claim 40 or 41 wherein the
diploid donor cell has been reprogrammed using a method
as claimed in any one of claims 37 to 39.

- 45 -

43. A recipient cell into which the reprogrammed
nucleus of a diploid cell has been introduced.

44. A cell as claimed in claim 43 wherein the recipient
5   cell is an oocyte.

Fig. 1 a Sperm dispersion, Nuclear reprogramming and Re-assembly of nuclear envelope in cell-free extracts (developing mouse embryos/embryonic Stem, ES cells)

*Sperm Nuclei + Extract A +ATP + Creatine phosphate (CP)

Sperm dispersion, release of sperm-specific protamines and initiation of nuclear envelope assembly

30°C, 1 h

Extract B +Mg^{+2} +ATP +CP

Reprogramming of sperm nuclear chromatin and DNA repair

30°C, 30 min

GTP + Glycogen

30°C, 1 h

Fusion of membrane vesicles and formation of discrete nuclear envelope
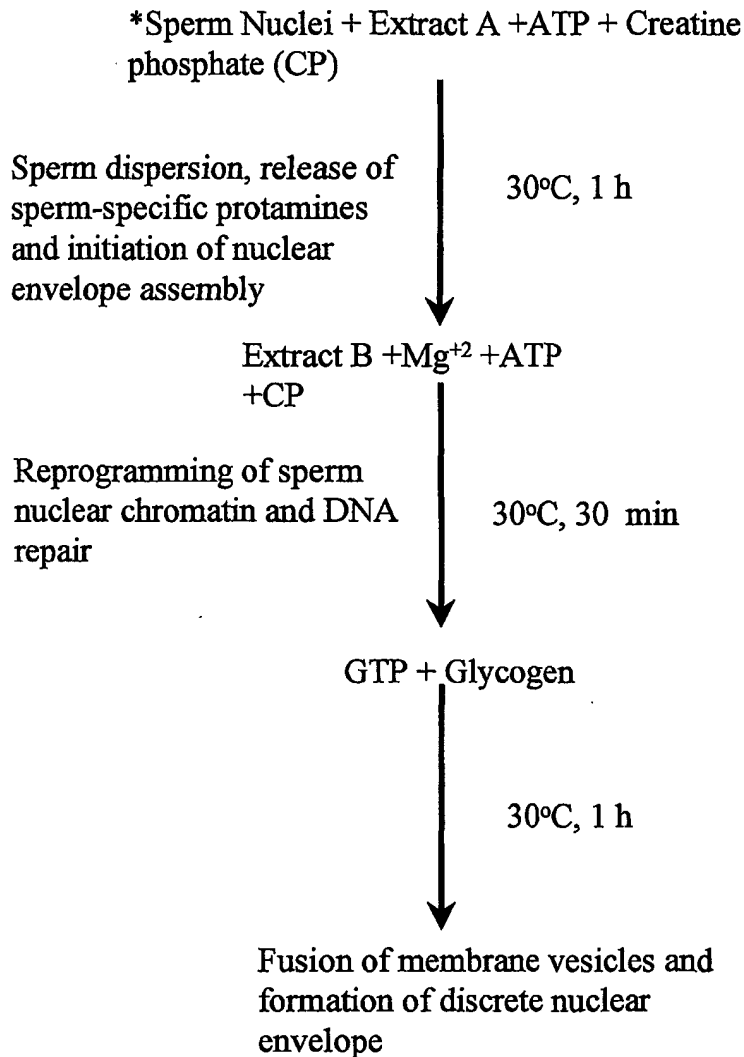
* Where sperm-mediated gene transfer (transgenics) is intended, the sperm nuclei are pre-incubated with a vector containing drug-resistant gene (neomycin, G418) and a colour selection marker gene (lac Z).

Fig. 1b   A schematic presentation of the methods of sperm-egg fusion, sperm-mediated transgenics and and diagnosis of human sperm chromosome abnormalities

Method A:
Fertilisation by fusion or microinjection

Human semen/Mouse epididymes

→ spermatozoa

→ Sperm Nucleus

→ Sperm dispersion, Nuclear reprogramming and reassembly of nuclear envelope in cell-free extracts (embryonic stem, ES cells)

→ Electrofusion of sperm nuclei with denuded zona-free oocytes or Microinjection into egg cytoplasm

→ Culture of embryos

→ Embryo Transfer

Method B:
Sperm-mediate transgenics in animals

Mouse epididymes

→ spermatozoa

→ Sperm Nucleus plus exogenous genes: coned genes or parts of chromosomes in micro-cells

→ Sperm dispersion, Nuclear reprogramming and reassembly of nuclear envelope in cell-free extracts (embryonic stem, ES cells)

→ Electrofusion of sperm nuclei with denuded zona-free oocytes or Microinjection into egg cytoplasm

→ Culture of embryos and assay for transgene expression

→ Embryo Transfer, Live birth and assay for transgene expression

Method C:
Diagnosis of Human Sperm Chromosome Abnormalities

Human semen

→ spermatozoa

→ Sperm Nucleus plus exogenous selectable markers (drug resistance)
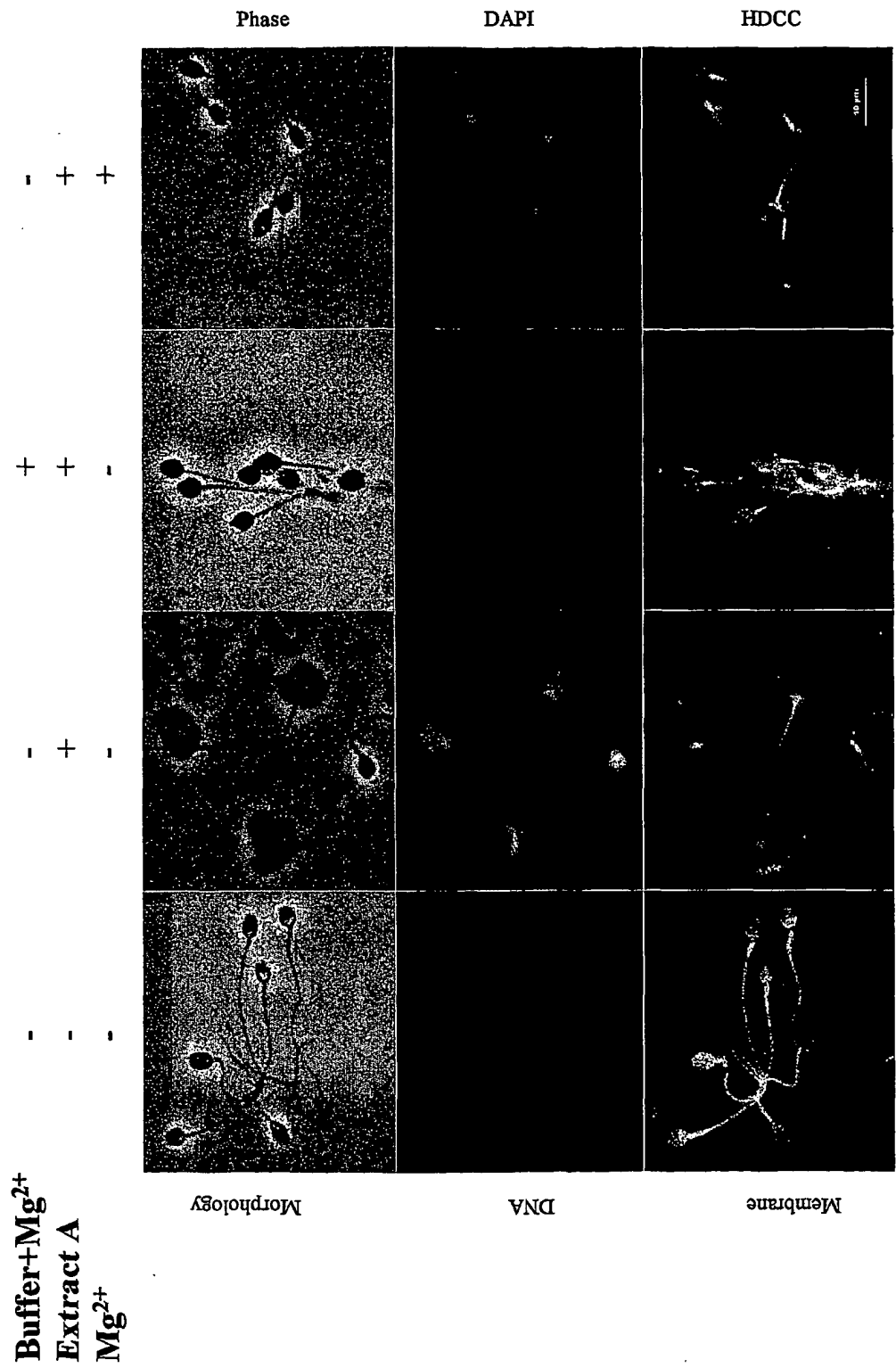
→ Sperm dispersion, Nuclear reprogramming and reassembly of nuclear envelope in cell-free extracts (embryonic stem, ES cells)

→ Electrofusion of sperm nuclei with Indian muntjac (Deer) or mouse ES cells

→ Selection of drug-resistant clones

→ Expansion of clones in the presence of colcemid or nocordazole

→ Cytogenetic analysis of human chromosomes by differential staining and painting
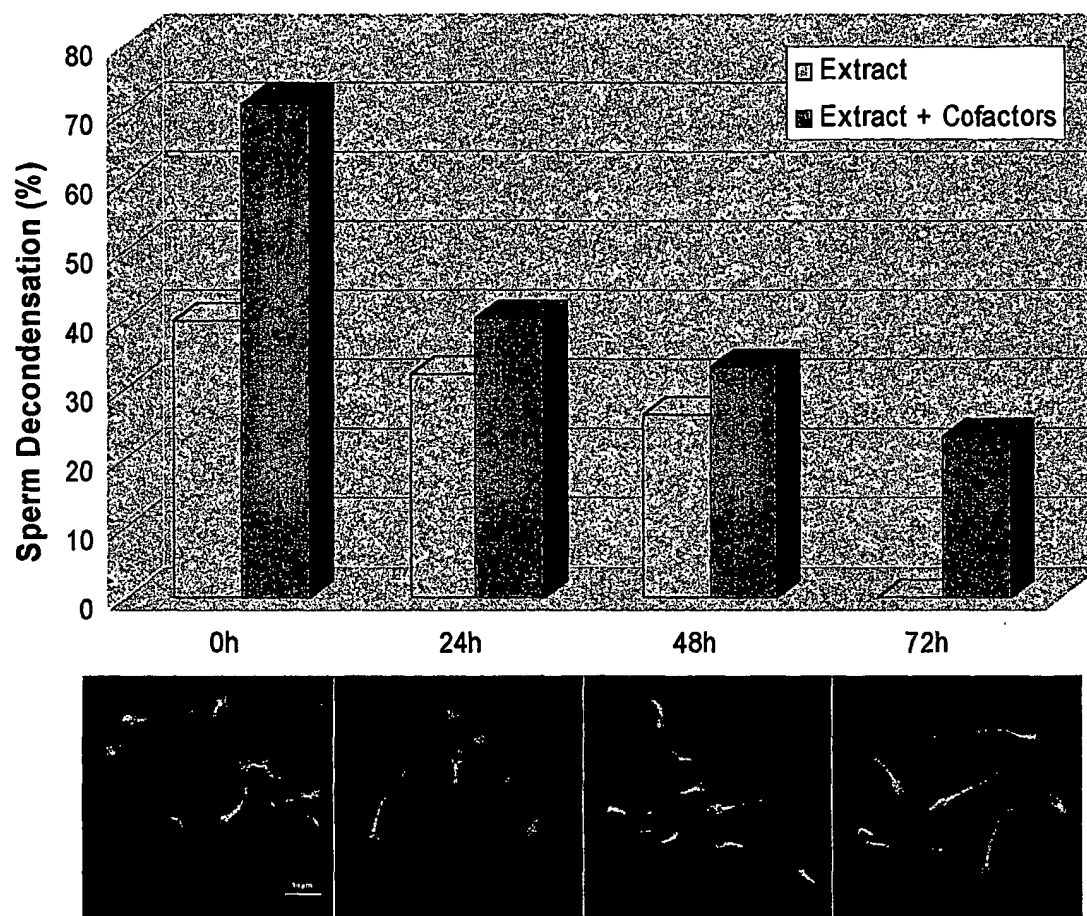
Fig.2   Inhibition of human sperm dispersion by Mg^{2+}.

Fig.3   Prolonged incubation of human sperm in IVF medium

(Medicult, UK) is detrimental to nuclear dispersion

Morphology   DNA   Membrane
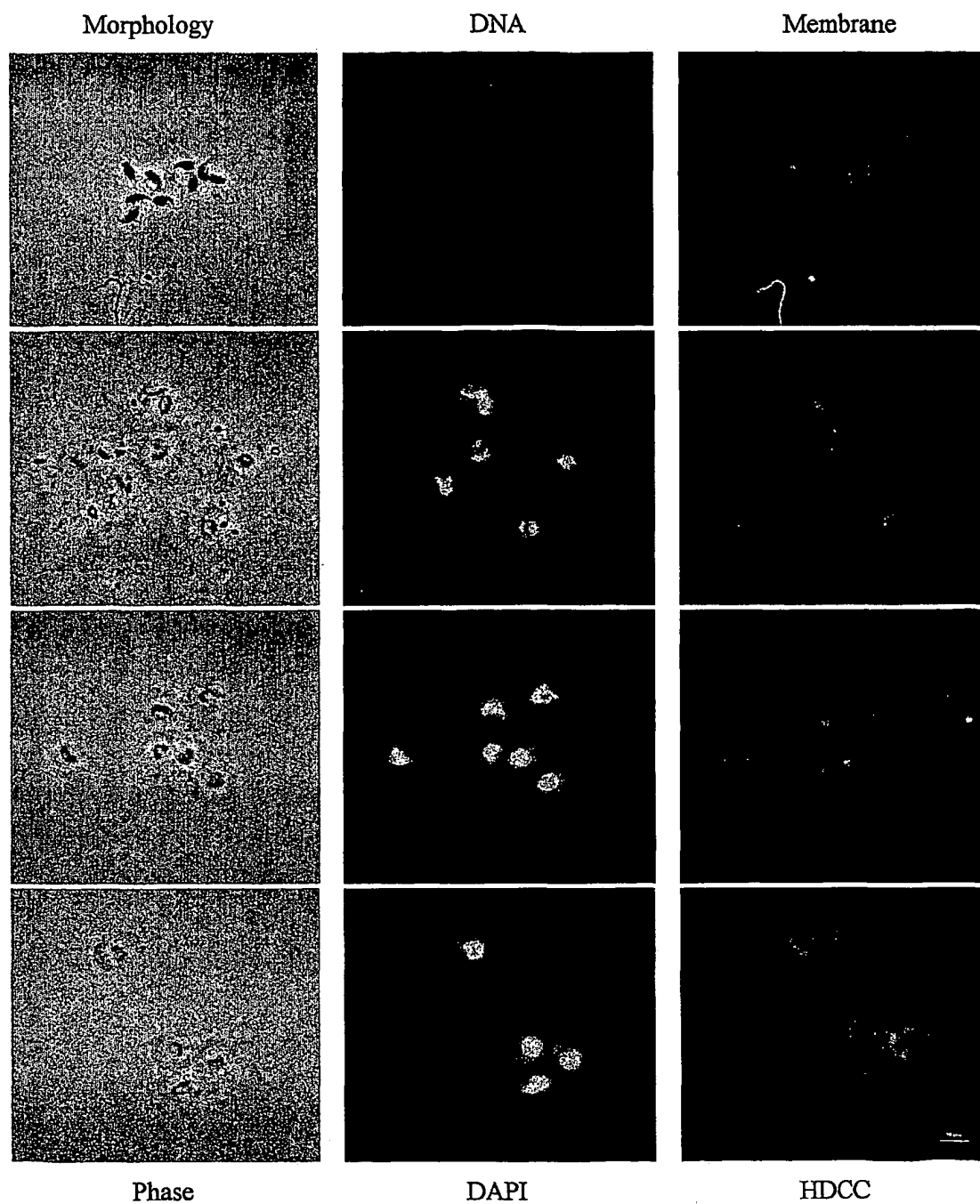

Phase   DAPI   HDCC

Fig.4 Mouse sperm dispersion, genomic reprogramming
and early stages of re-assembly of the nuclear envelope

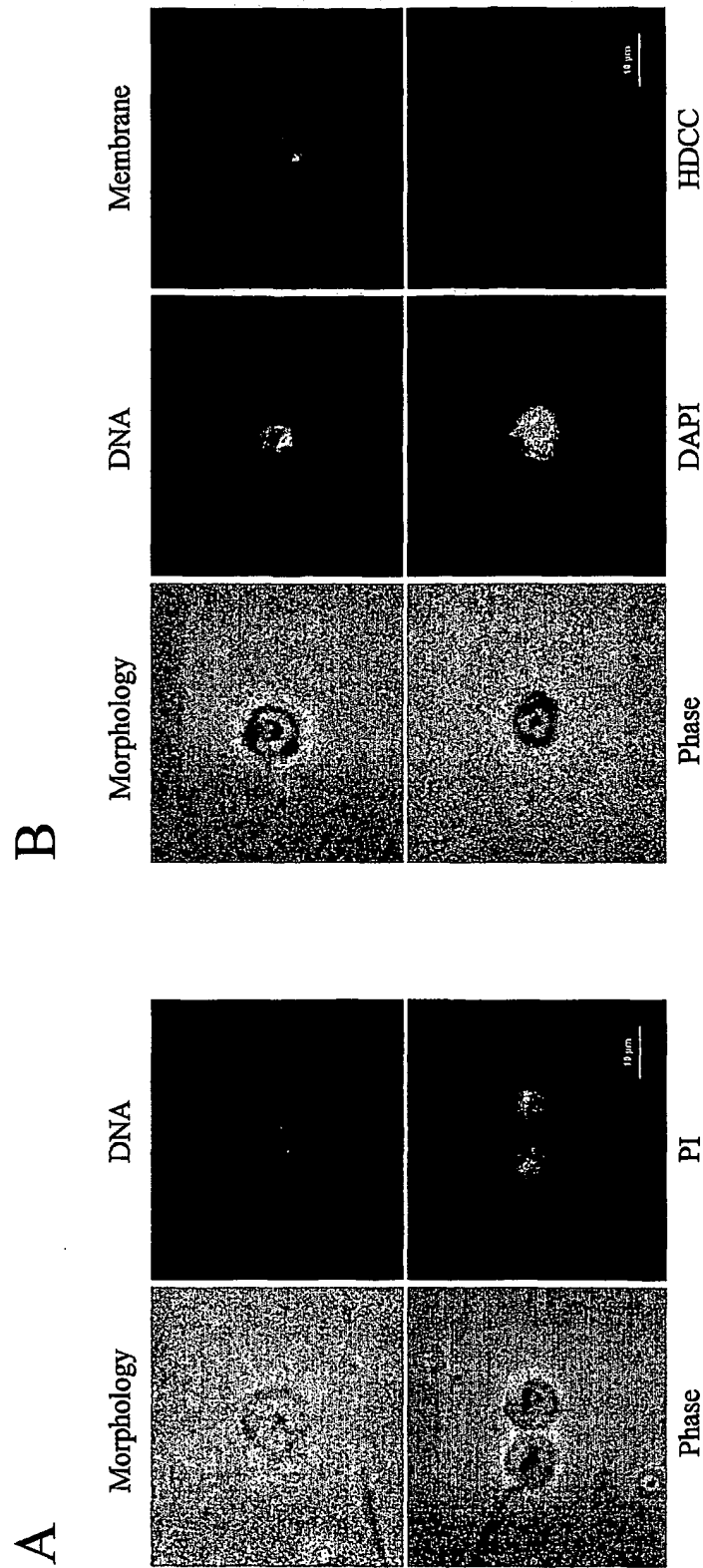Fig. 5 Mouse sperm nuclear size can be controlled following assembly.
A & B are results from two independent experiments

Morphology   DNA   Membrane

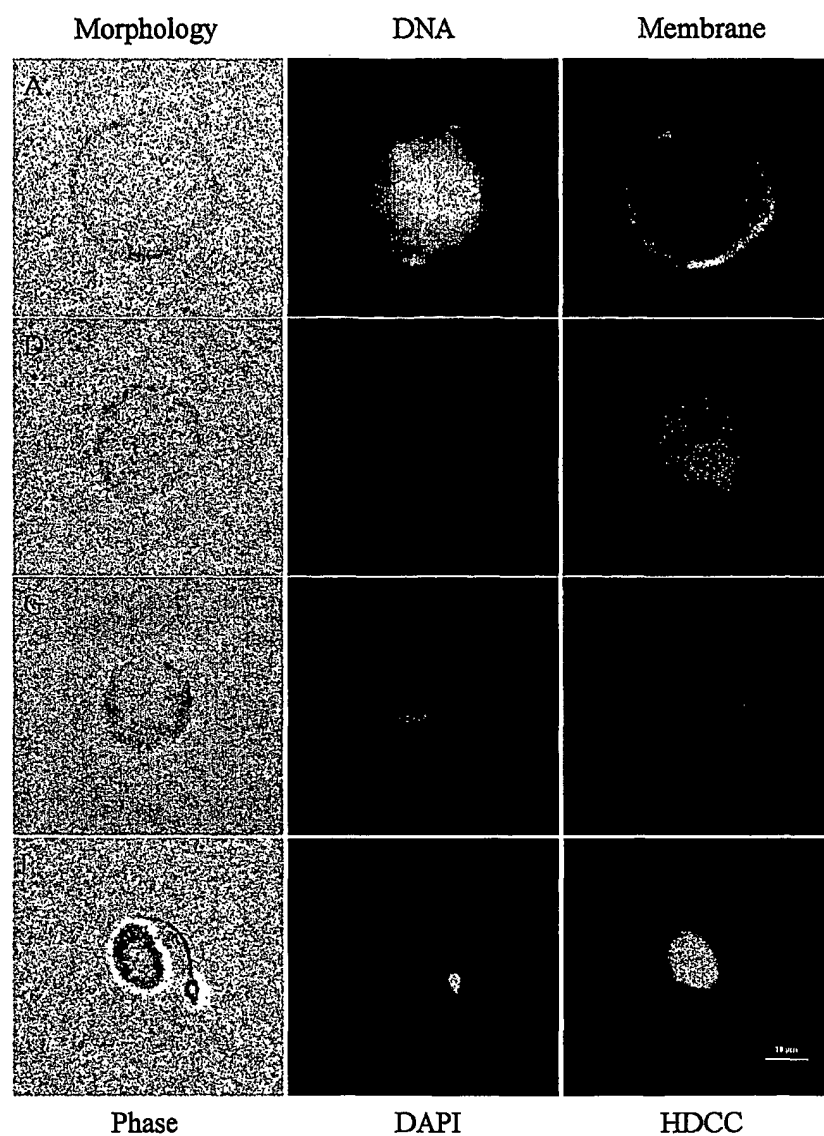Phase   DAPI   HDCC

Fig. 6 Human sperm dispersion, genomic
       reprogramming and reassambly of
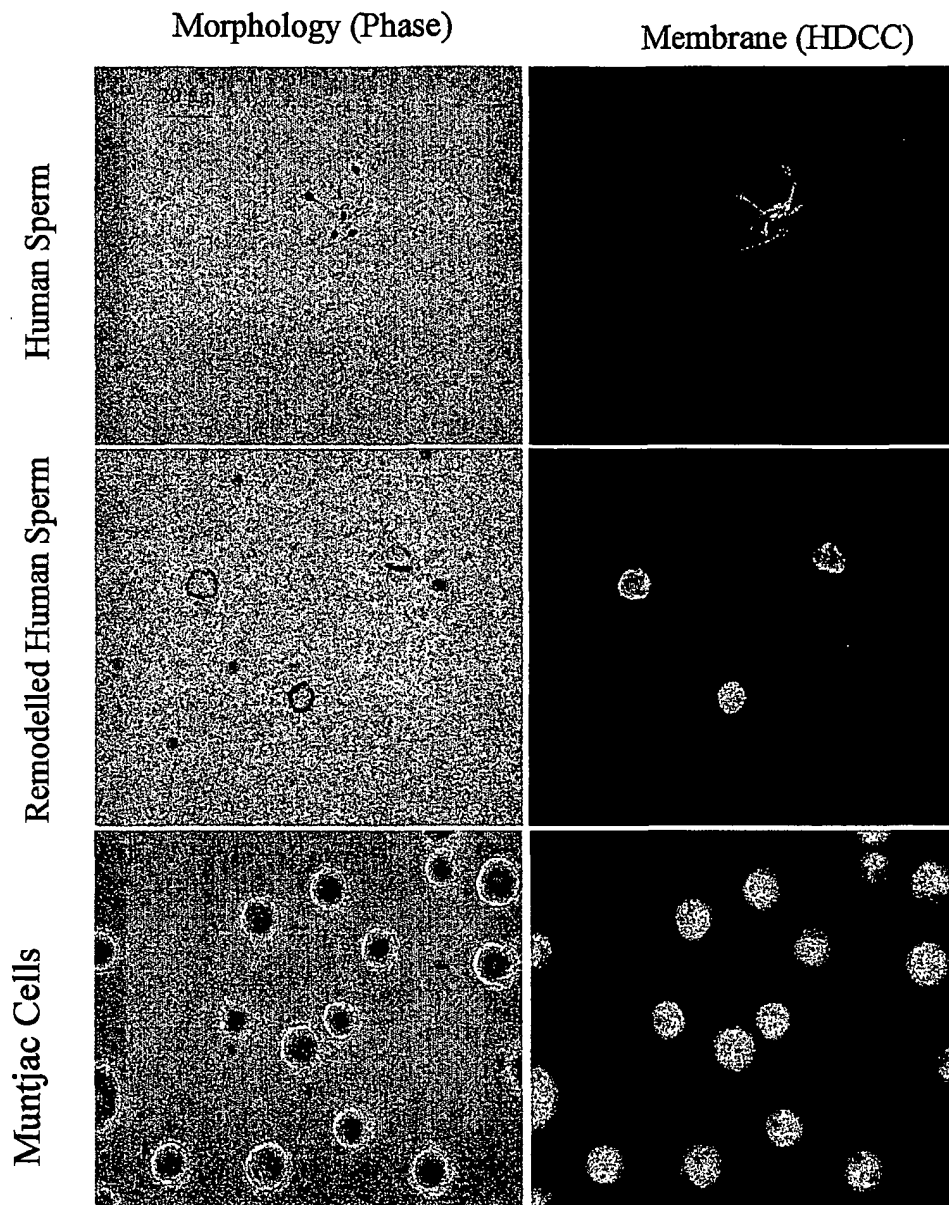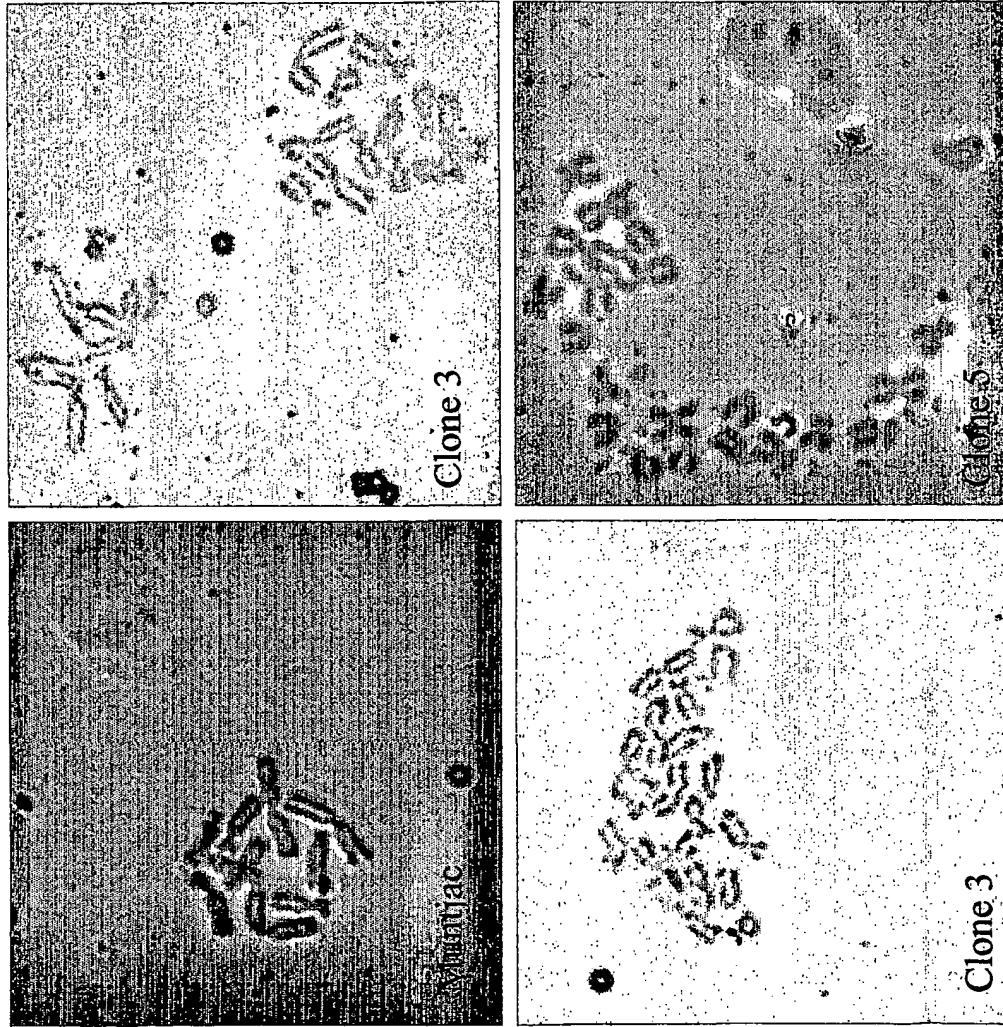   nuclear envelope.

Fig. 7. Morphological analysis of reprogrammed sperm nuclei and muntjac cells

Fig. 8. Chromosome composition of muntjac & hybrid cells
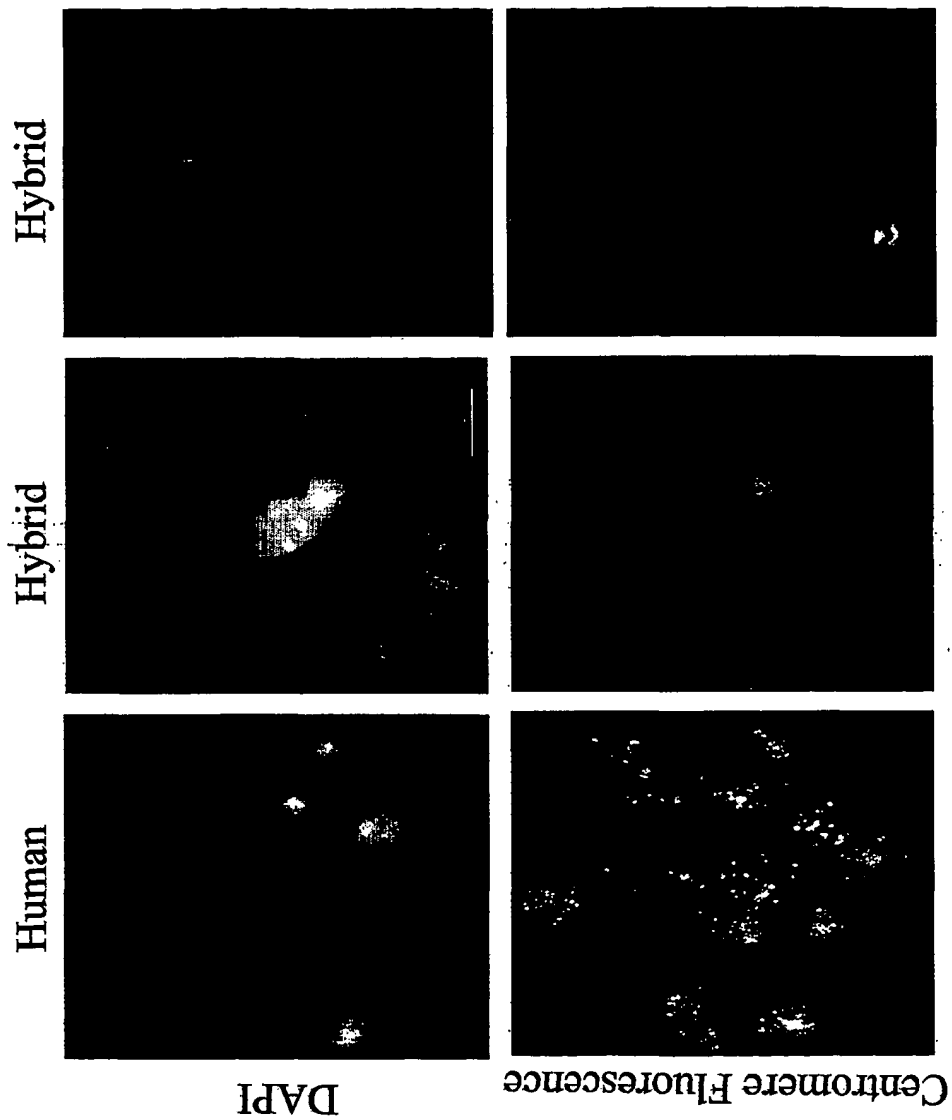selected for drug resistance after fusion with human sperm nuclei

Fig. 9. Demonstrates human chromosomes in muntjac hybrid cells

(51) **International Patent Classification**[7]: C12N 5/06, 5/02, 15/06, C12Q 1/02, C12N 13/00, A01K 67/02, A61D 19/00

(21) **International Application Number:** PCT/GB01/04184

(22) **International Filing Date:**
19 September 2001 (19.09.2001)

(25) **Filing Language:** English

(26) **Publication Language:** English

(30) **Priority Data:**
0022981.5   19 September 2000 (19.09.2000)   GB

(71) **Applicant** (for all designated States except US): ST. GEORGE'S ENTERPRISES LIMITED [GB/GB]; Cranmer Terrace, London SW17 0RE (GB).

(72) **Inventors; and**
(75) **Inventors/Applicants** (for US only): BANERJEE, Subhasis [GB/GB]; St. George's Hospital Medical School, Cranmer Terrace, London SW17 0RE (GB). NARGAND, Geeta [GB/GB]; St. George's Hospital Medical School, Cranmer Terrace, London SW17 0RE (GB). CAMPBELL, Stuart [GB/GB]; St. George's Hospital Medical School, Cranmer Terrace, London SW17 0RE (GB).

(74) **Agents:** GARDNER, Rebecca et al.; Frank B. Dehn & Co., 179 Queen Victoria Street, London EC4V 4EL (GB).

(81) **Designated States** (national): AE, AG, AL, AM, AT (utility model), AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ (utility model), DE (utility model), DK (utility model), DM, DZ, EC, EE (utility model), ES, FI (utility model), GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PH, PL, PT, RO, RU, SD, SE, SG, SI, SK (utility model), SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

(84) **Designated States** (regional): ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:
—   with international search report

(88) **Date of publication of the international search report:**
8 August 2002

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) **Title:** MANIPULATION OF SPERMATOZOA

(57) **Abstract:** The present invention relates to a method of sperm permeabilisation comprising: (a) washing a sample containing sperm in a sperm nuclear extraction buffer (SNEB) which includes one or more metal chelating agents and which is substantially free of non-ionic detergents and divalent cations; (b) contacting the sperm with one or more permeabilising agents; and (c) optionally separating the sperm tail from the sperm head; and to a method of sperm transformation which method comprises incubating a sample of sperm or sperm nuclei in one or more cell-free extracts. Also described are extracts and buffers for use in these methods, methods of preparing the extracts and uses of the transformed sperm nuclei.

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7   C12N5/06   C12N5/02   C12N15/06   C12Q1/02   C12N13/00
        A01K67/02   A61D19/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7   C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, BIOSIS, BIOTECHNOLOGY ABS, CHEM ABS, Data, EMBASE, LIFESCIENCES, MEDLINE, PAJ, SCISEARCH, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 5 651 992 A (WANGH LAWRENCE J) 29 July 1997 (1997-07-29) column 4, line 17 - line 67 column 5, line 56 -column 6, line 27 column 7, line 56 - line 65 column 9, line 52 - line 63 column 11, line 20 -column 13, line 30 column 15, line 36 - line 40 column 21, paragraph 4 column 23, line 65 -column 26, line 49 example 13	1-7, 9-18,27, 28
X	WO 95 21860 A (BANERJEE SUBHASIS ;HULTEN MAJ (GB)) 17 August 1995 (1995-08-17) cited in the application the whole document examples 1,2,4	1-7, 9-18,27, 28

-/--

X	Further documents are listed in the continuation of box C.	X	Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the International filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the International filing date but later than the priority date claimed

"T" later document published after the International filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed Invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed Invention cannot be considered to involve an inventive step when the document is combined with one or more other such docu- ments, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search

27 March 2002

Date of mailing of the international search report

15. 04. 02

Name and mailing address of the ISA
European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,
Fax: (+31-70) 340-3016

Authorized officer

Pilat, D

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X	COLLAS PHILIPPE ET AL: "Formation of the sea urchin male pronucleus in vitro: Membrane-independent chromatin decondensation and nuclear envelope-dependent nuclear swelling." MOLECULAR REPRODUCTION AND DEVELOPMENT, vol. 42, no. 1, 1995, pages 106-113, XP001034081 ISSN: 1040-452X cited in the application see Materials and methods section	1-7, 9-18, 25-27
X	LOHKA M J ET AL: "ROLES OF CYTOSOL AND CYTOPLASMIC PARTICLES IN NUCLEAR ENVELOPE ASSEMBLY AND SPERM PRONUCLEAR FORMATION IN CELL-FREE PREPARATIONS FROM AMPHIBIAN EGGS" JOURNAL OF CELL BIOLOGY, vol. 98, no. 4, 1984, pages 1222-1230, XP001037656 ISSN: 0021-9525 cited in the application see Materials and methods section the whole document	1-7, 9-18, 25-27
X	POCCIA D ET AL: "Transforming sperm nuclei into male pronuclei in vivo and in vitro." CURRENT TOPICS IN DEVELOPMENTAL BIOLOGY, vol. 34, 1996, pages 25-88, XP001038289 1996 Academic Press, Inc.;Academic Press Ltd. 1250 Sixth Ave., San Diego, California 92101, USA; 14 Belgrave Square, 24-28 Oval Road, London NW1 7OX, England, UK ISBN: 0-12-153134-1 the whole document	1-7, 9-18, 25-27
X	GORDON K ET AL: "IN-VITRO ACTIVATION OF HUMAN SERUM INDUCED BY AMPHIBIAN XENOPUS-LAEVIS EGG EXTRACT" EXPERIMENTAL CELL RESEARCH, vol. 157, no. 2, 1985, pages 409-418, XP002194533 ISSN: 0014-4827 the whole document	1-7, 9-18,27

-/--

INTERNATIONAL SEARCH REPORT

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X	SAWYER DENNIS. E ET AL: "The use of an in vitro sperm activation assay' to detect chemically induced damage of human sperm nuclei." REPRODUCTIVE TOXICOLOGY, vol. 9, no. 4, 1995, pages 351-357, XP002194534 ISSN: 0890-6238 abstract	7,9-17, 25,27,28
X	NEUBER EVELYN ET AL: "Efficient human sperm'pronucleus formation and replication in Xenopus egg extracts." BIOLOGY OF REPRODUCTION, vol. 61, no. 4, October 1999 (1999-10), pages 912-920, XP002194535 ISSN: 0006-3363 the whole document	7,9-18, 25,27,28
X	KAWASAKI KATSUMI 'ET AL: "Chromatin decondensation in Drosophila embryo extracts." JOURNAL OF BIOLOGICAL CHEMISTRY, vol: 269, no. 13, 1994, pages 10169-10176, XP002194536 ISSN: 0021-9258 the whole document	7-16,25, 27,28
T	LU PING ET AL: "Nuclear assembly of demembranated Xenopus sperm in plant cell-free extracts from Nicotiana ovules." EXPERIMENTAL CELL RESEARCH, vol. 270, no. 1, 15 October 2001 (2001-10-15), pages 96-101, XP002185219 ISSN: 0014-4827 see Materials and methods section	

INTERNATIONAL SEARCH REPORT

B x I   Observations where certain claims were found unsearchabl   (Continuation of item 1   f first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.: '
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II   Observations where unity of Invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

```
see additional sheet
```

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☒ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

```
completely 1-18,22,27-28 partially 25,26
```

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest   ☐ The additional search fees were accompanied by the applicant's protest.

☒ No protest accompanied the payment of additional search fees.

Form PCT/ISA/210 (continuation of first sheet (1)) (July 1998)

FURTHER INFORMATION CONTINUED FROM   PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. Claims: completely 1-6

method of sperm permeabilisation comprising a) washing a sample containing sperm in a buffer which includes one or more metal chelating agents and is substantially free of non-ionic detergents and divalent cations and b) contacting the sperm with one or more permeabilising agents.

2. Claims: completely 7-18, 22, 27-28, partially 25,26

method of sperm transformation comprising incubating a sample of sperm or sperm nuclei in one or more cell-free extracts, and sperm nucleus obtained therefrom.

3. Claims: completely 19, partially 25-26

method of preparing a sperm dispersion extract from a cell sample comprising a) suspending the sample in a buffer substantially free of divalent cations b) centrifuging the sample and c) collecting the supernatant, and kit containing said cell-free dispersion extract.

4. Claims: completely 20-21

method of preparing a sperm reprogramming extract comprising preparing a sperm dispersion extract from a cell sample comprising a) suspending the sample in a buffer substantially free of divalent cations b) centrifuging the sample, c) suspending the pellet product in a buffer and d) centrifuging the suspended pellet and f) collecting the supernatant product.

5. Claims: completely 23,24, partially 25-26

method of testing a sample of sperm or sperm nuclei comprising incubating the sample in a cell-free dispersion extract, and kits therefor

6. Claims: completely 29-32

method of in vitro fusion between a transformed sperm nucleus and a somatic cell or oocyte.

7. Claims: 33-35

method of testing for sperm chromosome abnormalities

comprising contacting a transformed sperm nucleus with a
somatic cell, causing the two nuclei to undergo fusion and
analysis of the chromosome, kits therefor.

8. Claim : 36

method of in vitro fertilisation comprising microinjection
of a transfused sperm nucleus into the cytoplasm of an
oocyte.

9. Claims: 37-39

method of reprogramming a somatic cell comprising incubating
said somatic cell in one or more cell-free extracts.

10. Claims: 40-44

recipient cell into which the reprogrammed nucleus of a
diploid cell has been introduced and animal embryo prepared
therewith.

Patent document cited in search report		Publication date	Patent family member(s)		Publication date
US 5651992	A	29-07-1997	US	5480772 A	02-01-1996
			US	6245567 B1	12-06-2001
			US	5773217 A	30-06-1998
			CA	2155309 A1	18-08-1994
			EP	0686202 A1	13-12-1995
			WO	9418344 A1	18-08-1994
WO 9521860	A	17-08-1995	AU	1585895 A	29-08-1995
			CN	1143967 A	26-02-1997
			EP	0743954 A1	27-11-1996
			WO	9521860 A1	17-08-1995
			JP	9510085 T	14-10-1997
			US	6187749 B1	13-02-2001